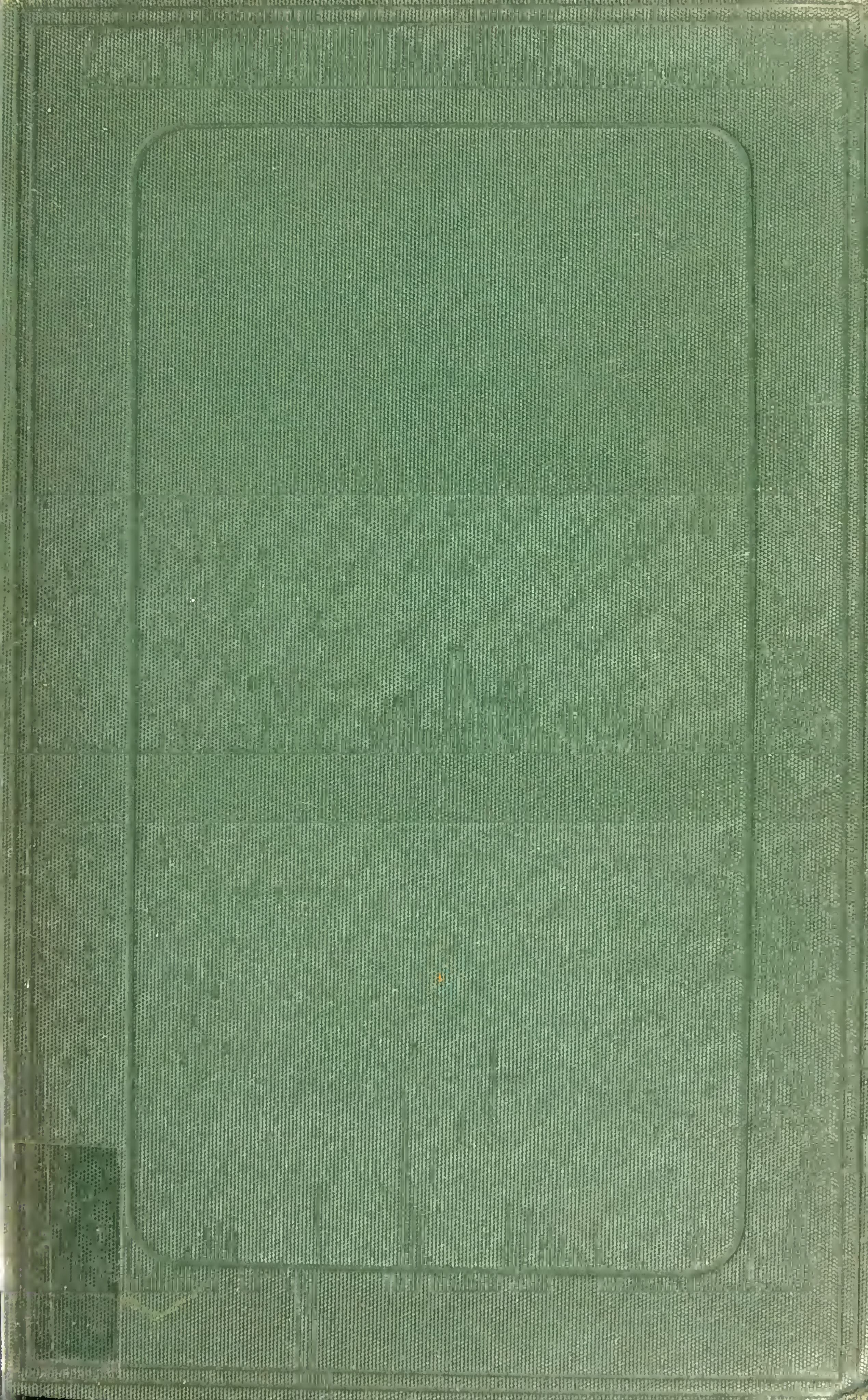

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METHODS OF PATHOLOGICAL HISTOLOGY

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METHODS
OF
PATHOLOGICAL HISTOLOGY

BY

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PREFACE

I TRUST that the translation of the third edition (1893) of Professor von Kahlden's *Technik der histologischen Untersuchen Pathologisch - Anatomischen Präparate,* which has long been regarded as a standard work on pathological methods in Germany, will be as generally useful in the hands of English pathologists who are not familiar with the original work. I take this opportunity of expressing my indebtedness to Professor von Kahlden for the courtesy and ready help he has afforded me during the progress of this translation, and especially for his consideration in furnishing me with the proofs of the last edition.

To my friend Dr. Rolleston for much help and advice in correcting the proofs, to Dr. Sherrington for several valuable suggestions which I have inserted as footnotes, and to Dr. Sims Woodhead, who has kindly written the Introduction, I must express my deep sense of obligation.

H. MORLEY FLETCHER.

HARLEY STREET, *January* 1894.

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INTRODUCTION

It is only within comparatively recent years that morbid histology has been considered of sufficient importance to require a knowledge of a special technique in its students. Continental workers were for long far ahead, in this respect, of our morbid histologists, and we then, as now, though in a lesser degree, depended upon them in great part for our knowledge of histological methods. All the earlier methods of hardening, embedding, staining, and mounting were worked out by the normal histologist, and many of them were not applied to pathological work for the reason that they were thought to be useful for the preparation of fresh tissues only. Closely following the histologist came the zoologists of the Naples school, who, under Dr. Dohrn, have introduced so many of our present methods for preserving and hardening delicate tissues.

Since the one great addition made by morbid histologists, the special methods of staining introduced and improved by Koch, Ehrlich, and Weigert, and their pupils, morbid histologists, and especially those working in the region of comparative pathology, have come to see that the best of the ordinary zoological, embryological, and histological methods can with great advantage be applied to morbid histology. The result of this has been that not only in the study of the nervous system and of bacteria in disease, but also in general pathology, the methods now at the command of the morbid histologist are as perfect as those used by other workers in the field of histology. Von Kahlden's excellent work on "Methods," first issued as a supplement to Ziegler's *Allgemeine und specielle Pathologische Anatomie*, and latterly in separate form, has for some time been well and most favourably known in the pathological laboratories in this country, and in introducing this English edition to a wider circle of readers, Dr. Morley Fletcher has conferred a real boon on the busy student of morbid histology who wishes to utilise, with as little expenditure of time and trouble as possible, the many useful methods contained in Von Kahlden's work. The translation, as those who are acquainted with the original work will find, is well done; but more important still, the translator has been able to give, in the form of footnotes, additions and modifications to the body of the work, the results of his own experience. These additions are many of them of great practical value, and greatly enhance the value of the book, which has by means of these additions been brought well up to date, and rendered, in its completed form, the most reliable and comprehensive work of its kind in our language.

GERMAN SIMS WOODHEAD.

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ERRATUM.

Page 143, line 6, for *dehydrate* read *decolourize.*

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CHAPTER I

THE MICROSCOPE AND APPARATUS REQUISITE FOR HISTOLOGICAL WORK

For ordinary work a high power magnifying 300 diameters, and one, or perhaps two, low powers are absolutely necessary. Two eye-pieces, a high and low, will be enough, the latter being most generally employed. The eye-piece magnifies the image formed by the objective, and not the object itself; any defects in this image therefore become more conspicuous with the higher eye-piece, and with it the illumination is not so good.

Apochromatic lenses are an exception to this rule, for much stronger eye-pieces can then be used, and greater magnification of the image produced, without any loss of definition.

Microscopes are generally supplied with both concave and plane illuminating mirrors. The concave mirror is the one generally used, while the plane is more adapted for working with low powers.

An oil immersion lens[1] is essential for the examination of bacteria. A drop of some oil, or of a mixture of different oils, which has nearly the same refractive index as glass, is placed between the objective and the upper surface of the cover-glass. In this way the air, which has a different index of refraction, is excluded. A soft silk rag, moistened with benzole, is used to remove the oil from the lens and from the cover-glass, provided that the Canada balsam has set and fixed the cover-glass.

Dust should be removed from the eye-pieces and objectives with a soft linen rag or camel's-hair brush. Any Canada balsam, cedar wood oil, etc., that may have dried on the surface of the cover-slip or objective should be removed with benzole. In doing so, care must be taken to avoid dissolving the Canada balsam, by which the lenses are cemented in the objective.

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[1] Water immersion lenses, which were the first form introduced, are now obsolete.

Opacities in the eye-piece can easily be detected by rotating it, and so be distinguished from those in the objective, which would remain stationary.

A condenser such as Abbé's must be used with the oil immersion. In this apparatus the rays of light coming from the mirror are collected by a lens, and brought to a focus.

As regards the choice of a microscope, it will be as well to select a stand which can be fitted later with an immersion lens and Abbé's condenser, should it be thought advisable not to procure them at first. Focusing by hand when using immersion lenses is out of the question. This is effected by the coarse adjustment (a wheel and rackwork) usually fitted to the larger stands.

Good and reliable instruments can be obtained from the following makers:—

C. Zeiss, Jena, Germany.
E. Hartnack, Potsdam, Germany.
E. Leitz, Wetzlar, Germany.
W. and H. Seibert, Wetzlar, Germany.

Instruments by these makers can be obtained through

R. Kanthack, 21 Golden Square, London.
C. Baker, 244 High Holborn, London.

Of the various English makers the following can be recommended:—

Powell and Leland, London.
H. Crouch, „
Swift and Sons, „
R. and J. Beck, „

The following objectives suffice for most purposes:—Objectives III. or IV. and VII. by Hartnack; II. and VII. by Leitz; I., III., and Va by Seibert; aa or A,AA and E by Zeiss. Of oil immersion lenses the 1/12 by Seibert or Zeiss[1] are perhaps the best.

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[1] For apochromatic lenses see the catalogues. Their high price prevents their employment for general purposes.

Illumination, etc.

Daylight is the best. Brilliant sunlight is quite unsuitable for any microscopical work. The light from a blue and cloudless sky is not very good, on account of its colour. The light from a white cloud, not too brightly lit up by the sun, is the most satisfactory.

In every microscope there is an arrangement by which the intensity of the light cast by the mirror on the specimen can be diminished at will by reducing the size of the opening, either in or below the stage. The drawback to the simple diaphragms often fitted to small microscopes is that they cannot be brought close up to the slide, and so do not shut off the diffuse light coming in from the sides.

There is no such disadvantage with the cylinder diaphragm. It can be pushed in from one side along a slide under the stage until it comes into the axis of the opening of the stage, and is then close under the specimen.

The "iris" diaphragm used with Abbé's illuminating apparatus is most useful. With this any gradation in the quantity of light admitted may be produced at will by a handle.

The definition of the elements in a section varies inversely as the illumination; the finer details disappear from view with a diffuse light in very transparent sections.

A small diaphragm should therefore be used with unstained, whereas a wider diaphragm may be employed for stained preparations.

In examining specimens stained for bacteria the brightest possible illumination is required. The diaphragm should have a wide aperture, or be dispensed with altogether, while the light should of course be concentrated by a condenser. Stained micro-organisms thus stand out more distinctly, since the definition of the surrounding tissue elements, which otherwise might easily conceal them, becomes almost, if not quite destroyed.

With artificial light the yellowish tint of the flame must be corrected either by means of a piece of blue glass placed between the lamp and the mirror, or by a glass globe containing blue ammonio-copper sulphate solution.

There are also several specially constructed microscope lamps.

In the lamp designed by Kochs-Wolz (Bonn), the light from a petroleum or gas flame is cast by a reflector on a fixed point of the black chimney which covers the lamp, and from thence is carried, by means of a bent glass rod, directly under the object, the mirror having been previously removed.

There are also a microscope lamp for gas or paraffin by Hartnack of Potsdam and a petroleum lamp of Dr. Lassar, to be obtained from F. W. Dannhause, Leipzig.

There are some additional appliances which may be required in microscopy.

1. A revolving nose-piece, *i.e.* a disc, into which from two to five objectives can be screwed. With this a given portion of the section can be rapidly examined under different powers, thus obviating any risk of mȯving the specimen. Lately a sliding arrangement for changing the objectives has come into use.

2. Micrometer eye-piece. This consists of a piece of glass, having a fine scale engraved on it, which is inserted into the cylinder of the eye-piece. It can either be slipped in from the side, or, after taking off the upper lens, can be dropped in so that it lies on a circular shelf inside the cylinder.

3. Drawing apparatus. The most useful are the camera lucidas of Abbé or of Seibert. By means of two prisms, a magnified image of the specimen is cast on a sheet of drawing-paper placed on a level with the stage. The exact outline of the specimen and of its individual parts can thus be easily reproduced. A raised drawing-board should also be provided, and should be constructed so that the paper is on the same level as the stage.

Lead pencils of different degrees of hardness are required for drawing. Etching pens with different coloured inks are often useful.

The warm stage, polariscope, and spectroscope are not required for ordinary purposes.

A dissecting lens may be wanted for making delicate preparations, as in teasing.

The slides should be of colourless glass. Slips of pasteboard may be fastened on them as a protection with shellac solution of about the consistency of syrup, on which the name or number of the specimens can be written.

The cover-glasses should not as a rule be thicker than 0·16 mm. Different sizes should be at hand, according to the sizes of the sections to be mounted.

Slides and cover-glasses should be cleaned by placing them in a vessel containing alcohol, and afterwards drying them with a soft linen rag.

The following will also be required:—

A number of watch-glasses of various sizes for staining and washing the sections, etc.; also some glass vessels which can be closed with a lid, to hold sections which are not going to be mounted immediately.

Lastly, glass rods, capillary tubes, test tubes, and bottles of different sizes.

Needles made of glass must be used when working with metallic solutions.

Steinach's "Siebdose"[1] (compound lifter) is useful when many sections have to be stained at a time (to be obtained from Siebert, Alsenstrasse, Vienna). This is a glass vessel, the floor of which is pierced with holes like a sieve, and which can be placed in several outer vessels consecutively, so that the sections lying on the sieve can be treated with various reagents in the outer vessels.

At present these vessels are capable of further improvement, since the feet of the sieve are too high and the perforations are too far apart.

Glass apparatus, etc., can be obtained from

Baird and Tatlock, 14 Cross Street, Hatton Garden, London.
R. Kanthack, 21 Golden Square, London.
Becker, 33 Hatton Wall, London, etc.

The following metal instruments will be required—Needles, forceps, scissors, scalpels, lifters, etc.

The needles must be quite smooth and free from any roughness, otherwise delicate sections will have a tendency to adhere to them. If the needles become roughened they should be rubbed with fine glass paper, and afterwards polished on a piece of leather. Needles which can be screwed into a holder are the most convenient, as when one becomes spoilt a fresh one can be inserted in its place.

A blunt curved silver needle will be found very useful in all manipulations of sections, such as lifting and transferring them, or removing them from the knife of the microtome.

A "lifter" is used for transferring the sections from one fluid to another, especially from water into alcohol, and also for spreading them out on the slides. The best are made of thin platinum foil, of any size desired, and may even be made large enough for exceptionally big sections, such as those of the brain.

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[1] To be obtained through Kanthack, Golden Square, London.—Ed.

Sections cannot be so readily taken off or spread out on the thicker brass lifters, besides which these are liable to be acted upon by various reagents.

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CHAPTER II

EXAMINATION OF FRESH TISSUES

For examining microscopically fresh tissues which have been removed from the body either before or after death, the specimen is generally prepared by the process of teasing. A very small piece is cut out with forceps and a small pair of curved scissors, and is then teased out as finely as possible with two needles on a slide in a drop of water or of 0·6 per cent salt solution.

Salt solution has this advantage over water, that in it the tissue does not swell so much, and the finer structures undergo less alteration. Serous fluids, blood serum, hydrocele fluid, etc., have the same advantage.

An artificial serum can be prepared by mixing nine parts of salt solution with one part of egg albumen. In it the cells of the tissue retain their normal appearance much longer.

In examining for micro-organisms in fresh tissues it must be borne in mind that bacteria can develop not only in distilled water, but even more readily in salt solution. Micro-organisms which are found in the tissues may therefore have their origin in the solution, especially if it be no longer fresh.

A small drop only of the fluid should be added. With even a slight excess the cover-glass will not lie firmly, and swims about, or the free cells in the preparation will exhibit movements which very much interfere with their examination. Excess of the solution also prevents or delays the action of other reagents which it may be desirable to use.

Teasing is chiefly used in the examination of muscle, brain, spinal cord, and those tumours which have an abundant interstitial substance. In examining nerves and muscles the excised piece must be small, and should contain only short portions of the nerve or muscle fibres, as they are very difficult to separate when long.

The dissociation of fresh preparations into particles sufficiently small and delicate to be examined under a microscope is often very tedious. When this is the case, the small pieces are placed for twenty-four hours in a dissociating fluid, and after this they can be readily teased into the smallest fragments. As a rule, when portions of the organs of an animal are to be examined by a method of dissociation the pieces should be placed in the selected dissociating fluid immediately after the animal is killed. Pieces of human organs should also be exposed to the action of the fluid in as fresh a condition as possible.

The dissociation is carried on in small watch-glasses; the volume of the fluid should not greatly exceed that of the tissue, otherwise hardening rather than dissociation will result.

The following **Dissociating fluids** are useful:—

1. 33 per cent **Alcohol** ("one-third Ranvier's alcohol"), made by mixing one part 96 per cent spirit with two parts distilled water.

2. Dilute **Chromic acid** solutions (0·01 to 0·03 per cent) are very useful in many cases.

3. **Osmic acid** solution, 0·1 per cent.—It requires twelve to twenty-four hours to act, and dissociates very well. This is especially adapted for tissues containing fat.

4. **Arnold's method.**—The tissue is placed for five to ten minutes in 0·1 per cent acetic acid, and then for twenty-four to twenty-eight hours in 0·01 per cent chromic acid. It can afterwards be treated with picro-carmine, etc.

5. 33 per cent **Caustic potash.**—The tissues break up in this within an hour. It is very useful in the examination of unstriped muscle fibres, such as those in tumours of the uterus, etc. For the microscopical examination, 33 per cent potash solution must also be used, because if it be diluted with water the cells are destroyed.

6. **Müller's fluid** (*vide* p. 13) can also be used for dissociation, especially in the case of the nervous system. Pieces of tissue should remain in it from two to three days. When using a dissociating fluid the microscopical examination should be made in a drop of the same liquid, though water or salt solution can be used.

Besides teasing, the method of scraping may be employed in the examination of fresh tissues. The freshly-cut surface of the organ is scraped with the blade of a knife, and the juice of the tissue which adheres to it mixed with a drop of water or salt solution. The blood must first be removed from the cut surface by gently scraping and washing it, as otherwise the numerous red blood corpuscles will obscure everything else in the specimen. Care must also be taken to avoid placing too much of the scraping on the slide. The mixture should be rendered only slightly turbid. This method is especially applicable to glandular organs, particularly the liver and kidney when examining for a granular or fatty change. It also applies to tumours, especially carcinomata, but also to soft sarcomata,[1] and lastly, to inflammatory exudations, as, for example, those of the lungs.

In the examination of fluids, in most cases a drop may be placed at once on a slide. If the fluid contains very many cells or other solid constituents, as is the case with cystic fluid, contents of the stomach, etc., it is advisable to take up a drop of the fluid by a fine pipette, or on a platinum loop, and to dilute it with an equal quantity of water or salt solution. On the other hand, when the fluid contains very few cells, it must be allowed to settle for some time before the examination can be successfully undertaken.

The centrifugal process lately advocated by Litten is an excellent method for separating a sufficient amount of material for examination from fluids which are poor in cells.

We may also mention that the method of drying a film on a cover-glass (comp. p. 79) and afterwards staining it, may be employed in the investigation of all fluids.

The examination of **fresh tissues** can in all cases be facilitated by the addition of various reagents, which have the property either of rendering the preparation more transparent, or else of making certain parts stand out more distinctly, while others are not affected by it, or may even become fainter. Of these reagents the following are in general use:—

1. **Glycerine.**—In addition to its property of rendering tissues more transparent, it has the advantage of neither evaporating nor of undergoing chemical changes. Preparations, therefore, which are treated with it can be preserved if the entrance of air

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[1] And also squamous-celled epitheliomata; *vide* Butlin, *Sarcoma and Carcinoma*, p. 154. Butlin shows that scrapings from epitheliomatous ulcers of the tongue contain epithelial cells differing from those obtained from tuberculous and syphilitic and other ulcers in the following points: The cells are of very variable size and shape, with nuclei much larger than normal, and always contain a quantity of granular material. Cell-nests may also be observed.—Ed.

be prevented. Pure glycerine is generally used, though it is occasionally diluted with water. Fresh preparations are rendered so very transparent by it that it is only suitable for the examination of pigment. On the other hand, glycerine is often employed in the case of unstained sections which have been prepared from previously hardened material.

2. **Potassium acetate** as a saturated solution (50 per cent) may be used for clearing tissues, but is less powerful than glycerine, so that fresh sections may be examined in it. Permanent specimens can be mounted in it, as in glycerine.

3. **Acetic acid.**—This has a double advantage: it causes the nuclei to shrink, and makes their outlines more distinct, and produces a swelling of the connective tissues, which therefore become more transparent.

Fat molecules withstand the action of acetic acid, while, on the other hand, the granules seen in cloudy swelling are dissolved. Acetic acid is on this account an excellent reagent for differentiating between fatty degeneration and the granules of cloudy swelling. Micro-organisms are unaffected by acetic acid. Elastic fibres are unaltered by it, and after its use stand out more distinctly in contrast to the fibrous tissues, which become swollen and transparent.

Acetic acid is generally used as a 1 to 2 per cent solution, made by diluting glacial acetic acid with water. As a rule, a drop of the dilute acid is applied by means of a glass rod to one side of the cover-glass covering the preparation, which should have been previously examined. The acetic acid slowly penetrates, and its action can be followed under the microscope. A small piece of blotting-paper placed on the opposite side of the cover-glass so as to absorb the fluid causes the acid to flow in more quickly.

Fuchsine-acetic acid brings nuclei very prominently into view, both by sharpening their definition and by staining the nuclear substance. It is prepared by adding fuchsine to a 2 per cent acetic acid solution until the colour becomes a deep red.

Lastly, the acetic acid solution is used to dissolve the lime salts out of tissues which have undergone calcareous changes.

4. A weak solution of **Iodine** in water is also a very useful reagent for defining the nuclei and details of the tissues. This solution is prepared by diluting the ordinary Lugol's solution (iodine 1 part, potassium iodide 2, water 100) with water until it is of a light yellow colour.

5. **Caustic potash or soda.**—The effects produced by this reagent are very different, according to the strength of the solution.

The 1 to 3 per cent caustic potash solution dissolves most tissues. The following are the only important structures which resist its action—Elastic tissue, fat, pigment, and micro-organisms, which in this way can be easily differentiated. Lardaceous or amyloid material also resists a weak caustic solution, but this property is seldom made use of in microscopical examinations, as there are specific reactions for lardaceous tissues.

A 33 per cent strong caustic potash or soda solution does not destroy the cell constituents of connective tissue; on the contrary, it dissolves the cement substance which binds them together, and is on this account employed to dissociate the cells of tumours, smooth muscle fibres, convoluted glands, etc. Dissociation is generally effected in a few minutes. After this the tissues should not be treated with water, as if this be done the caustic solution will be diluted, and the action of the weaker solution takes place in addition. The examination must therefore be carried on in the strong caustic solution.

6. **Osmic acid.**—This is used as a 1 per cent aqueous solution, and may be added to the specimen, previously teased out and placed under a cover-glass, in the same way as the acetic acid solution. It stains fat molecules from a brown to a black colour, and is therefore a valuable reagent for showing the presence of fat, especially in fatty degeneration.

7. **Hydrochloric acid,** 3 to 5 per cent solution.—This is used in the examination of calcifications, chalky concretions, etc. It dissolves out the phosphate of lime, so that the parts which were previously dark now become clear. As the carbonate of lime dissolves, bubbles of gas (CO_2) make their appearance.

8. **For staining fresh tissues.**—If they have not been already stained as dried cover-glass preparations, watery stains, such as methyl green, or Löffler's methylene blue (*vide* p. 79), or fuchsine-acetic acid (*vide* p. 10), may be run in from the edge of the cover-glass (*vide* p. 10). Hæmatoxylin is not suitable.

CHAPTER III

HARDENING TISSUES

For the more minute examination of tissues, and when thin sections are required, some preliminary process of hardening is necessary.

It is most important that the pieces of tissue be placed in the hardening fluid as soon as possible after death, or after their removal from the living body, in order to prevent decomposition changes.

1. **Alcohol** is the hardening fluid in most general use. The strength used is from 90 to 100 per cent (usually 99 per cent). It is advisable, to prevent shrinkage, to place the tissues first in 90 to 96 per cent spirit, and on the next day in absolute alcohol. Many tissues are hardened quite sufficiently in ordinary 96 per cent spirit. Moreover, the whole of the water contained in 96 per cent spirit can easily be removed by placing in it freshly ignited copper sulphate. This salt should be heated in a metal crucible until it is converted into a white powder. When it has turned blue again in the spirit, fresh powder should be dropped in, or that already used should be re-ignited. The alcohol which has been rendered absolute in this way should be filtered, otherwise particles of the copper salt may be deposited in the tissues.

For more delicate work it is better to use absolute alcohol, because this is chemically purer than spirit which has been dehydrated by artificial means.

The pieces to be hardened in alcohol are cut into cubes or slices, not more than 2 to 3 cm. in thickness. The volume of alcohol should be ten to fifteen times that of the pieces of tissue. The alcohol must be changed on the second, fourth, and, if necessary, also on the sixth days, and replaced by fresh. The bottle should also be shaken occasionally during the first day, as otherwise pieces may adhere to the bottom, so that the alcohol cannot act on their under surface.[1]

Alcohol can be used as a hardening agent for most tissues, and is especially useful when a rapid examination is required. It is far preferable to other hardening agents when micro-organisms are suspected. To prevent any subsequent changes of the micro-organisms in the tissues, absolute alcohol should be employed from the first. Speaking generally, alcohol is not suitable for hardening tissues from the nervous system.

To preserve entire organs and tumours for collections, they should be placed in a stream of water, to get rid of the blood which they contain, until the water which flows away is quite clear. They are then placed in 70 to 80 per cent alcohol, which is repeatedly changed until no further turbidity appears.[2]

2. **Müller's fluid** :—

Potassium bichromate .	2·5	per cent.
Sodium sulphate . .	1	„
Distilled water . .	100	„

It requires at least twelve weeks at ordinary temperatures to fully harden small pieces in this fluid, and for large organs, such, for example, as an entire brain, as long as a year. The hardening process can be somewhat hastened by placing the tissue for a time in an incubator. In this case, however, the hardening fluid must be frequently changed.

The volume of the fluid should be ten to twenty times that of the pieces to be hardened. It should be changed on the second, fourth, sixth, and twelfth days, and whenever it becomes turbid or infected by mould. Tissues can be kept in Müller's fluid for as long as ten years, but after the lapse of a year half its volume of water should be added to the fluid. Should the tissues require further hardening, they should be washed for some hours in water, which is repeatedly changed, and then placed in 30 per cent spirit for one day, and then in 96 per cent. If a precipitate is produced in the latter, the spirit must be changed. Tissues can be kept for several years longer in spirit, but after a time they cease to stain properly. Müller's fluid has the following advantages over alcohol:—

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[1] Pieces of cotton wool placed at the bottom of the bottle effect the same result, and the bottle should be well shaken as well.—Ed.

[2] It is better to keep them permanently in a solution of distilled methylated diluted to "proof" by the addition of distilled water.

(*a*) Tissues shrink less in Müller's fluid, and the cells and interstitial substance are better preserved.

(*b*) Red blood corpuscles preserve their shape excellently in Müller's fluid, and retain their yellow colour, which is not the case with alcohol.

(*c*) As a rule, tissues which have been hardened in Müller's fluid stain better than those which have been preserved in alcohol, especially with hæmatoxylin which is so generally used; this, however, is only the case if the pieces have been in the Müller's fluid for a sufficiently long time; otherwise the staining is much inferior to that obtained in alcohol preparations.

(*d*) The trifling cost of Müller's fluid as compared with alcohol.

Alcohol and Müller's fluid amply suffice as hardening reagents for ordinary requirements.

3. **Erlicki's fluid.** Composition:—

Potassium bichromate .	2·5 grms.
Copper sulphate . .	0·5 „
Water . . .	100·0 ccm.

The advantage of this fluid is that tissues can be hardened in eight to ten days, or in four to five days in an incubator. It is inferior to Müller's fluid in that the tissues are more liable to shrink, and precipitation may occur.

4. **Corrosive sublimate**[1] is an excellent hardening agent in certain cases, which will be referred to later. As well as hardening the tissues, it "fixes" the figures of indirect nuclear division. It is made by saturating a warm 0·5 per cent salt solution with corrosive sublimate (= 7·5 per cent). The pieces, which should not be too thick, should remain in this solution for one to two hours, rarely as long as six hours. They are then thoroughly washed in water for twenty-four hours, and hardened in 30 per cent, 70 per cent, and 96 per cent alcohol, twenty-four hours in each. Thorough washing is absolutely necessary after hardening in sublimate to prevent mercurial precipitates being produced in the tissues, which in the subsequent examination might

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[1] Preparations hardened in corrosive sublimate stain much better with the various carmine dyes than with hæmatoxylin.—Ed.

be mistaken for pigment. It is easy to ascertain, by the addition of a drop of iodine solution, if there is still any sublimate present in the washings, in which case a yellowish-red precipitate of iodide of mercury will be produced. It has been recommended to add tincture of iodine to the 70 per cent alcohol which is used for hardening afterwards, until it acquires a light yellow colour. The iodine removes the excess of corrosive sublimate by the formation of iodide of mercury, and the alcohol changes colour. Corrosive sublimate is in general less suitable for hardening post-mortem specimens than for those removed from the living body.

5. **Picric acid** is used as a saturated aqueous solution. Small pieces, not more than 1 cm. thick, are left in it for twenty-four hours, and are then transferred to 70 to 80 per cent spirit. It must be borne in mind when using picric acid as a hardening agent that it also removes the lime salts from bony tissues and calcareous structures. Sections of tissue hardened in picric acid must be very carefully washed, or else they will retain the yellow stain.

6. **Osmic acid** is used for hardening purposes as a 1 per cent aqueous solution. Owing to the fact that osmic acid penetrates very imperfectly into the tissues, the slices of tissue should not be more than 5 mm. thick. They remain in the solution for twenty-four hours, and are then placed in 80 per cent spirit. Osmic acid stains fat particles black, and on this account is very useful for showing their presence. For hardening tissues which have undergone fatty degeneration, *vide* p. 63.

Sections treated with osmic acid should be preserved in potassium acetate solution (*vide* p. 10), because glycerine gradually becomes brown. The darkening of the glycerine can, however, be prevented by leaving the sections for a few days, before they are finally mounted in it, either in water or in diluted glycerine.

7. **Flemming's osmic acid solution** is a very useful hardening agent in many cases. For method of application, *vide* pp. 56 and 64.

8. **Glycerine and gum,** on account of the very rapid and thorough hardening which it produces, may be used in many cases in which there is no need for great histological detail.

The glycerine and gum solution is prepared by adding to boiling glycerine as much powdered gum arabic as will dissolve in it, the mixture being kept continually stirred.

Pieces of tissue, which have been freshly removed from the body, are first freed from blood in running water. Tissues which have been previously kept in spirit are washed in water to remove the alcohol.

They are then transferred to the gum and glycerine solution for twenty-four hours, being kept under the surface of this by means of glass rods, etc. After twenty-four hours they are put into 80 to 90 per cent spirit, and the vessel well shaken at intervals. In a few hours the pieces are hardened and ready to cut.

Before staining, the sections should be well washed in plenty of water, which must be changed, in order to dissolve the gum which has been precipitated by the alcohol.

9. **Boiling** may be employed in cases in which the tissues contain albuminous fluids, which coagulate on heating, such as occur in œdema of the lungs, nephritis, and in cysts. A small cube of the tissue, 1·5 ccm. thick, is placed in boiling water for one to two and a half minutes, and is afterwards hardened in 96 per cent spirit.

CHAPTER IV

THE DECALCIFICATION OF TISSUES

THE lime salts must be removed from bones and such tissues as contain calcareous or osseous material before sections can be cut. The following points require attention :—

1. The tissues in question must be well hardened before they are placed in the decalcifying fluid, otherwise the soft parts of the tissues will be much altered. They may be hardened in alcohol, but preferably first in Müller's fluid, and subsequently in alcohol.
2. The fluid used for decalcifying should be abundant and frequently changed.
3. When the decalcification is completed the pieces must be very carefully washed in water for at least two days, so that no trace of the decalcifying fluid remains, as it afterwards damages the tissue.
4. After the pieces of tissue have been thoroughly washed they must be again hardened; they are then ready to be cut.

The time required for complete decalcification depends not only on the strength of the fluid employed, but also largely on the thickness and size of the pieces. Moreover, the bones of new-born children, and more especially fœtal bones, are much more rapidly decalcified than those of adults. The pieces selected for decalcification must not be too large. To ascertain whether the lime salts have been completely removed, the piece should be pricked with a needle, or a trial slice cut with a scalpel.

The following are the decalcifying solutions in most general use :—

I. **Chromic Acid and its Salts.**

Müller's fluid decalcifies to a slight extent, but is only applicable for small pieces of bone, or fœtal bone. The time required for it to decalcify more than this is almost always too long. Its action can be hastened by Haug's method, in which 1 ccm. nitric acid is added to 200 to 300 ccm. of the fluid every time the fluid is changed, that is on the third, fifth, and eighth days. One advantage of Müller's fluid is the excellent way it preserves the tissues.

A 2 per cent solution of pure chromic acid also requires a long time to decalcify; it acts somewhat more quickly when used as a mixture of

Chromic acid . .	1·0 grm.
Nitric acid . .	1·0 ccm.
Water . . .	100·0 „

According to Haug, this gives very good results with young bones.

Waldeyer recommends the following chromic acid method:—

1. Fix the fresh tissue in chromic acid 1 in 600.
2. Harden in chromic acid, first in 1 in 40 and afterwards in 1 in 200.
3. Decalcify in a mixture of
    Chromic acid (1 in 200) 100 ccm.
    Pure nitric acid, 2 ccm.

The solution should be changed every six days.

Then wash thoroughly and harden in alcohol. It takes three months or longer to decalcify in this way.

Fol's decalcifying solution has the following composition:—

Chromic acid (1 per cent) = 70 parts.
Nitric acid = 3 „
Water = 200 „

For very delicate tissues **Haug** recommends the following solution:—

1 per cent osmic acid . .	10 ccm.
1 per cent chromic acid .	25 „
Distilled water . .	65 „

Wash in water and harden in 70 per cent alcohol.

II. Saturated Solution of Picric Acid.

An aqueous solution containing an excess of picric acid is the best. It acts simultaneously as a hardening, fixing, and staining agent.

The tibia of a new-born child placed uncut in this solution is decalcified in about three weeks. Larger and older bones require several months. It acts more quickly if 3 to 5 per cent nitric acid be added. Sulphuric acid cannot be substituted, as sulphate of lime is thereby produced, which is nearly insoluble.

III. **Lactic Acid,** as a 10 per cent or stronger solution.

This, like picric acid, removes the lime salts somewhat more quickly than chromic acid, and only slightly affects the tissue.

IV. **Pyroligneous Acid** (Acid. Pyrolignosum pur.).

Haug strongly recommends this in an undiluted solution for fœtal bones, for those of the lower animals, and also for bony and cartilaginous tumours. It also hardens to a certain extent. It is not so suitable for treating adult bones.

V. Hydrochloric Acid.

This is generally employed in Von Ebner's decalcifying solution.

It has the following composition :—

Sodium chloride . .	2·5	grms.
Hydrochloric acid . .	2·5	ccm.
Alcohol . . .	500·0	„
Distilled water . .	100·0	„

Von Ebner's solution acts more slowly, but is more effectual than the 1 to 10 per cent solution of hydrochloric acid formerly in use. The process can be hastened by increasing the amount of hydrochloric acid to 5 per cent, and raising proportionately the quantity of chloride of sodium used.

Tissues embedded in celloidin, which have not been properly decalcified, may be treated again with Ebner's fluid. In this way the superficial layers at least become more fully decalcified.

Before cutting, the acid must be removed by washing in 70 per cent spirit.

Waldeyer recommends the following solution of **Palladium chloride** :—

Palladium chloride . . .	0·01 ccm.
1 per cent hydrochloric acid solution .	1000·0 „

After being thoroughly washed, the tissue is hardened in 30 per cent, 60 per cent, and 90 per cent spirit successively.

VI. Pure Nitric Acid.

This is used diluted in the proportion of 3, 5, to 9 parts to 100 parts of water. It is specially suitable for adult bones.

Simple solutions of nitric acid alter the relations of the structures less than the corresponding solutions of hydrochloric acid. In order to avoid the long delay required in soaking in an aqueous solution, Haug recommends the following alcoholic solution :—

Sodium chloride . .	2·5	grms.
Pure nitric acid . .	30·0 to 90·0	ccm.
Absolute alcohol . .	700·0	„
Distilled water . .	300·0	„

This solution decalcifies quickly and well. It may be used equally for fœtal, young, or adult bones. Care must be taken to fix the tissues previously in corrosive sublimate, as they then retain their powers of staining very well after all the other hardening processes.

VII. Phloroglucin Method.

This is a special modification of the nitric acid decalcifying solution. Phloroglucin itself does not dissolve out lime salts, but it protects the tissues from the action of the acids which are used in combination with it. Hence the acids may be employed in a more concentrated form, and the decalcification of small pieces of bone may be completed in half an hour, or, in the case of harder bony material, within a few hours.

When this method is employed the process of decalcification must be watched continuously.

The solution is prepared as follows :—

Phloroglucin 1·0 grm. is dissolved with careful warming in 10·0 grms. of pure, non-fuming nitric acid.

100 ccm. of a 10 per cent solution of nitric acid is added to this ruby-red solution.

If a larger quantity of the solution is required a larger quantity of the phloroglucin solution should be freshly prepared.

The following decalcifying fluid is somewhat slower in its action :—

Phloroglucin . . .	1·0 grm.
Nitric acid . . .	5·0 ccm.
Alcohol	70·0 „
Distilled water . . .	30·0 „

VIII. **Thoma's Method.**

1. Harden in Müller's fluid or in alcohol.
2. Decalcify in the following solution, which should be repeatedly changed :—

   96 per cent spirit, 5 volumes.
   Pure strong nitric acid, 1 volume.

3. Wash in spirit.
4. Transfer to spirit, which should be changed several times, and to which an excess of precipitated calcium carbonate has been added.

Decalcification of pieces of bone of larger size is completed in two to three weeks. To prevent the calcium carbonate adhering to them, the pieces are wrapped in blotting-paper. It takes fully eight to fourteen days to remove the acid in the spirit containing the chalk powder. The pieces should be left in this for a few days even after no free acid can be detected with litmus paper.

CHAPTER V

METHODS OF EMBEDDING

SOME tissues even after careful hardening become only moderately firm, and require embedding before they can be cut into sections. Some embedding materials need not be removed from the sections before staining or examining them. Their use is to fix certain structures or abnormal constituents of the tissues so that they do not fall out in the various processes to which the sections may later be subjected.

Embedding in Celloidin.

Celloidin fulfils both these conditions better than any other material, because it remains quite transparent in the sections and most stains can be washed out of it. Two solutions of celloidin, a thin and a thick, are required, the latter being of the consistency of thick syrup. They are prepared by dissolving celloidin, cut up into very small pieces, in a mixture of equal parts of alcohol and ether.

The tissue must have been previously quite dehydrated in absolute alcohol, and it is unnecessary after this dehydration to place it in a mixture of alcohol and ether, as is often recommended. After they have been in the alcohol, the pieces, which should not be more than 1 cm. thick, are placed in the thin celloidin solution for twenty-four hours, and then in the thick solution for the same length of time. In dealing with preparations which are difficult to manipulate, and also in all cases in which time is no object, it is advisable to leave the pieces in both celloidin solutions for several days, as the embedding is more successful the longer they are left in these solutions.

If the pieces are afterwards to be cut by a freezing microtome they are taken out of the thick celloidin and placed on a glass slide, while they are at once placed on a piece of cork if an ordinary sliding microtome is employed, to which the cork holding the preparation is attached by means of a clamp. An additional quantity of the thick celloidin is poured over the preparation, whether it be on a glass plate or on a cork, should sufficient not have adhered originally.[1] The preparation, with the celloidin surrounding it, is allowed to partially dry in the air, and is then immersed for twenty-four hours in 80 per cent alcohol, which hardens the embedding material. The degree of solidity which the preparation acquires thereby, depends very materially on whether it was dried quickly or slowly in the air. When dried slowly it becomes much firmer. If the piece is to be cut in the frozen condition a half-hour's exposure to the air before placing it in the 80 per cent spirit will produce a sufficient degree of firmness. Otherwise the pieces are apt to become too hard when frozen, and do not adhere to the plate of the microtome.

If the other form of microtome (*vide* p. 33) is employed the preparation should be dried under a bell-glass as slowly as possible with exclusion of the air. The most satisfactory method is to place the preparation under a bell-glass, not simply with the celloidin which adheres to it, but to surround the cork with a cylinder of stiff paper fastened to it with a pin. Into the little chamber formed in this way a thin layer of celloidin is poured, and the tissue placed therein. More celloidin is now poured over it until the piece is thickly covered. In this way it is possible to harden it quite slowly within one to three days, as the tissue is surrounded by a considerable mass of celloidin, and preparations treated in this way become quite as firm as those embedded in paraffin. Preparations, such as pieces of membranous tissue which will not remain by themselves in a given position, should be fixed by means of pins stuck loosely into the cork. The ring of paper should be removed when the preparation has solidified in alcohol. The block of celloidin is then cut into a four-sided figure, which is the best shape for the microtome.

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[1] Caldwell recommends that after passing the piece of tissue through the two solutions of celloidin in the usual way, it should be placed in a paper boat and surrounded by the thick celloidin solution. The paper boat is then floated on the surface of chloroform. This causes the celloidin to set rapidly and without shrinkage, and to acquire an excellent consistency for cutting. The vessel containing the chloroform should be covered to prevent evaporation. If the tissue is to be cut by the freezing microtome, it is next placed in 90 to 95 per cent alcohol, then in water, to remove the alcohol, and finally in gum solution.

To embed subsequently in paraffin, transfer the tissue surrounded by celloidin from the chloroform to benzole, add the paraffin, heat on a water-bath, and finally place in pure hard paraffin (see also p. 26).—Ed.

If it be desirable to retain the layer of celloidin around the separate sections, they must not remain too long in absolute alcohol during the process of dehydration, as after some time the celloidin dissolves. After staining and washing the sections, they are placed in 96 per cent spirit, which removes the greater part of the water. Complete dehydration can then be effected by quite a short stay in absolute alcohol.

With the same object Nikiforoff recommends the addition of an anhydrous fluid which does not dissolve celloidin, and which mixes well with alcohol, and uses for this purpose a mixture of equal parts of absolute alcohol and chloroform. For clearing the sections, bergamot, cedar, or origanum oil can be used, while clove oil must be avoided, as it dissolves the celloidin at once. It is often necessary, particularly when using aniline colours, to completely remove the celloidin from the sections before staining. They should then be taken out of the water or dilute spirit in which they were placed after removal from the knife of the microtome, and transferred to absolute alcohol. After this they are put for ten to fifteen minutes in a mixture of equal parts of alcohol and ether, or in oil of cloves, then back again into absolute alcohol, and lastly, into water. They are now ready for staining.

As shown above, the process of embedding in celloidin can be divided into the following stages:—

1. Hardening in absolute alcohol.
2. One to five days in a thin celloidin solution.
3. One to five days in a thick celloidin solution.
4. Drying in the air on a cork or on a glass plate.
5. Twenty-four hours in 80 per cent spirit.
6. Cutting.
7. Staining and washing.
8. Dehydration in 96 per cent alcohol, followed by one to two minutes in absolute alcohol.
9. Clearing in bergamot, cedar, or origanum oil, or in xylol.
10. Canada balsam.

Embedding in Paraffin.

Embedding in paraffin has many distinct disadvantages as compared with embedding in celloidin, and is therefore seldom employed for pathological preparations.

In the first place, the prolonged exposure of the preparations to a temperature of 50° C. is a tedious process. Secondly, the paraffin must be removed from the sections before mounting, and thereby one of the chief advantages of embedding is lost, namely that of fixing in their places loose connective tissue constituents, exudations, deposits, etc. On the other hand, the very convenient method of staining in bulk when paraffin is employed is not generally used for pathological purposes.

The process is as follows :—

The hardening process must be completed in absolute alcohol. The tissue is then placed for twenty-four hours in some solvent of paraffin, the best of which, besides ethereal oils, is xylol. It is next heated to 50° C. in a mixture of paraffin, which melts at 50° (different paraffins having different melting points). When quite saturated with the liquid paraffin, the tissue is placed in a small cardboard box, or in an enclosure made by laying together on a glass plate two pieces of metal bent at right angles, and is fixed in this trough in the position desired. The position of the preparation must be accurately noted, as it will afterwards be impossible to see how it lies in the solidified and opaque block of paraffin. The solidification of the paraffin can be hastened by pouring cold water upon it. The block of paraffin thus produced is then placed in the proper position for cutting, fixed in the clamp of the microtome, and is cut without moistening the knife.[1]

To remove the paraffin, the sections are placed in xylol,[2] after this in alcohol, and then in water.

Lastly, if the pieces have not already been stained in bulk, the sections are placed in the staining fluid.

The process in paraffin embedding is accordingly divided into the following stages :—

1. If not already hardened in absolute alcohol, it must be passed through this reagent.
2. Twenty-four hours in xylol.
3. One to twelve hours in an oven at 50° C. in melted paraffin.
4. Pour out and allow it to solidify.

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[1] Turpentine is often used instead of xylol. For cutting a ribbon, the block of hard paraffin, the edges of which have been pared, is covered with a thin coating of soft paraffin. This connects the successive sections into a continuous ribbon. The rocking microtome, made by the Cambridge Scientific Instrument Company, is generally used in this country for cutting sections in paraffin.—Ed.

[2] See also footnote, p. 43.

5. Cut with the microtome.
6. Xylol.
7. Alcohol.
8. Water, staining, etc.

As a rule, sections embedded in paraffin are too brittle to stand without further precautions the various processes required in staining and mounting. Each section should be fixed on a slide. For this purpose a very thin layer of oil of cloves and collodion (three to one) is smeared on the slide. The section is fixed to it, and the whole is then heated for five to ten minutes over a water-bath or in an oven at 60° C. It is then placed in xylol, in order to completely remove the paraffin. The section is then ready for mounting in Canada balsam should the tissue have been previously stained in bulk before cutting. If not already stained the slide is transferred from the xylol to 96 per cent spirit, and then successively into water, staining fluid, water for washing, alcohol, xylol, Canada balsam. There are a number of other cements besides the clove oil and celloidin mixture, of which the following may be mentioned:—

Solution of egg albumen.—This is prepared by beating up into a froth a measured quantity of egg albumen and then adding an equal volume of pure glycerine. This is filtered,[1] and a very thin layer spread on the microscope slide. The section, having been placed upon it, is heated for a short time either over a water-bath or in an oven at 60° C. in which the solution sets and firmly attaches the section to the slide.

Shellac solution.—A concentrated solution of white shellac in absolute alcohol is made up, and a very thin layer of it spread on the warmed slide with a glass rod. A section is pressed on the shellac, and the slide is then placed in a vessel and exposed to the vapour of a small quantity of ether which is poured on the bottom.

Afterwards—water-bath or warm oven—xylol to remove the paraffin, etc., as above.

Celloidin and Paraffin Methods combined.[2]

By combining the celloidin and paraffin methods of embedding, tissues may be prepared so that they cut excellently; large and thin sections can be thus obtained.

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[1] It is better to filter before adding the glycerine, as the latter filters so slowly. Camphor or salicylic acid should be added to preserve.—Ed.

[2] See also p. 23.

The method is as follows:—

1. Transfer the piece of tissue from alcohol to a mixture of equal parts of absolute alcohol and ether for some hours.
2. Saturate for twenty-four hours in a moderately thick solution of celloidin.
3. Transfer to origanum oil.
4. Place it in a mixture of paraffin and origanum oil, which should not be heated higher than 40° C.
5. Place in melted paraffin.

The further treatment is the same as in ordinary paraffin embedding.

Other Methods of Embedding.

The methods of embedding in soaps of various composition and in mixtures containing egg albumen are hardly ever employed. Celloidin is used instead.

There are, however, some methods which may be of use for a rough and rapid examination.

Small objects, particularly membranous tissues, can be quickly cut by squeezing them between two pieces of hardened ox or lardaceous liver. This is done by dividing a piece of lardaceous liver, placing the object to be cut between the two halves, and making sections through the liver either by hand with a razor, or with a small cylinder microtome, thus producing at the same time a section of the tissue to be examined.

Dutch cheese may be used for the same purpose; it should be kept for some time in 95 per cent spirit at 25° C.

Elder-pith can be employed in like manner; a piece is divided and the tissue to be cut placed between the two halves. The pith is then immersed in water, in which it swells up and firmly surrounds the tissue. This may also be cut either by hand or with a cylinder microtome.

Another simple method of embedding is as follows:—

The piece of tissue to be cut, which should be only a few millimetres high, is placed on a cork in a large drop of gum solution. The cork is then immersed in 96 per cent alcohol, in which the gum sets and firmly surrounds the tissue.

CHAPTER VI

INJECTION OF TISSUES

By this is meant the artificial filling of the vessels with some pigment in a fluid condition. The process is made use of to show up more distinctly blood-vessels, especially those of the capillary system, which, under normal circumstances, are almost invisible; also for lymphatics, and for the ducts of glands with their ramifications. The method, however, is not employed so often in morbid as in normal histology.

The colouring fluid must not be injected too forcibly, or else the walls of the vessels may easily be ruptured and artificial extravasations produced. The pressure must be as uniform as possible. With a certain amount of practice great alterations of pressure may be avoided even when using ordinary syringes. The process, however, is somewhat tedious. The syringe must be well made, and the piston should slide up and down easily. A stop-cock should be fitted between the nozzle of the syringe and the cannula which is introduced into the vessel, so that there is no danger of injecting bubbles of air when the syringe is replaced after its removal for refilling. Cannulæ of different bores will be required. The cannula is tied into the trunk of the artery, and the injection continued until a sufficiently deep coloration is produced, or until the injection fluid has been flowing out of the vein for some time.

It often happens at the commencement of the injection that the fluid comes out from ruptured superficial vessels, especially in the veins under the capsule of the kidney. Such vessels should be closed by means of pressure-forceps if there be much loss.

An apparatus for injecting under a constant pressure, capable of being increased or diminished at will, can be easily constructed out of two bottles, provided there be a water-supply at hand.

The first of these bottles (A), which is hermetically closed, has a glass tube reaching to the bottom connected by a pipe with the tap of the water-supply. Another glass tube, bent twice at right angles, leads from the top of this bottle into the second bottle (B), into which it opens close under the stopper. This bottle (B) is just like an ordinary wash bottle, and is fitted with a second glass tube bent twice, one end of which reaches to the bottom, the other end being attached to the cannula by means of an india-rubber tube.

The second bottle (B) is fitted with the injection fluid. A certain amount of water is allowed to run into the bottle (A) from the water-pipe, so that the compressed air in A, by pressing on the fluid in B, causes this to flow out.

If no water-supply be available its place must be taken by a third bottle (C), which stands on a higher level, and is filled with water or mercury. This bottle C communicates with the bottle A by an india-rubber tube, which can be closed by a clamp.

By opening this clamp any quantity of water or mercury desired runs into the bottle A, and compresses the air therein in the same way as that from the water-pipe.

It is hardly necessary to say that when the bottle (C) is filled with water it must stand much higher than when filled with mercury.

The material used for injection may be either one which remains fluid when cold, prepared by suspending the colouring matter in water or glycerine, or one to which glue has been added so that it is liquid when heated and solid at ordinary temperatures.

The solid injection gives the better results, but is more difficult to use, because the injection material, as well as the tissue to be injected, must be kept at a temperature of 40° to 50° C. This is best effected in water at the required temperature. Of the numerous injection fluids which have been recommended the following may be mentioned :—

1. Soluble Berlin blue . .	1·0 grm.
Distilled water .	20·0 ccm.

2. **Cohnheim's injection fluid.**

Aniline blue . .	1·0 grm.
0·5 per cent salt solution	600·0 ccm.

3. **Thiersch's method** with Berlin blue and glue.

First prepare—

A, a cold saturated solution of sulphate of iron.
B, a cold saturated solution of red prussiate of potash.
C, a saturated solution of oxalic acid.
D, a solution of glue in the proportion of 2·1.

15 grms. of D are mixed with 6 ccm. of A in a porcelain dish. In a second dish 30 grms. of D are mixed with 15 ccm. of B, and then 12 ccm. of C added to it.

Both dishes are heated to 30° C., and the contents of the first added drop by drop to the second, constantly stirring it all the time.

Then the whole of this deep blue mixture is heated to 75° to 100° C., and filtered through flannel on a hot-water funnel.

4. **Kollmann's carmine injection fluid.**

1 grm. of carmine is dissolved in a little water with 15 drops of strong ammonia, and diluted with 20 ccm. glycerine.

To this is added a mixture of 30 grms. glycerine and 1 grm. common salt. The whole is then diluted with an equal quantity of water.

Excellent injection fluids ready for use may be obtained from Dr. Grübler, 12 Bayersche Strasse, Leipzig.

To hasten the solidification of the glue mixture, the injected tissue is placed in cold water, and subsequently in 80 per cent alcohol.

Organs injected with a mixture which is fluid when cold should be placed in spirit at once, and after a few hours cut into moderate-sized pieces.

Mention must also be made of the method of injecting lymphatics by puncturing the tissues. A fine cannula is cautiously driven in at the spot chosen. It is often best to puncture through the wall of a vessel into the surrounding tissue. Then the lymph spaces and lymphatics which are thus penetrated are injected under very carefully regulated pressure.

CHAPTER VII

PREPARING AND MOUNTING SECTIONS—CUTTING IN SERIES

Good microscopic sections may be obtained from fresh as well as from hardened tissues. They are usually prepared from the latter, because they can then be cut thinner and more uniformly, stain better, and make better permanent specimens. As many features of the tissues change in the hardening process, the examination of sections from the tissues when fresh, either in water or in 0·6 per cent salt solution, or by some of the methods previously given (p. 7), should also be employed.

In some cases the sections may be cut by hand with a hollow-ground razor. The edge of the blade should be straight and not curved.

The piece to be cut is held between the thumb and index finger of the left hand, so that it projects somewhat above the radial side of the forefinger. A smooth surface is then obtained, and from this the sections are cut as thinly as possible.

The blade of the razor is held at its juncture with the handle between the thumb and index finger of the right hand. The blade is then drawn somewhat obliquely across towards the preparation, and care must be taken only to use the minimum necessary pressure. The cutting should be done by drawing, not by pushing the edge.

More generally, uniform sections can be obtained, and the operation of cutting is facilitated, by resting the edge of the blade on the radial surface of the left index finger which is holding the preparation, and which thus serves as a support.

The razor should be kept continually moistened with water when dealing with fresh tissues, or with weak spirit for hardened specimens. The sections are taken from the blade, and placed in a wide and shallow dish containing either water or dilute spirit, as the case may be.

Microtomes.

By the use of a microtome larger and more perfect sections can be obtained. The following are the forms of this instrument which are in most general use:—

1. **The cylinder microtome.** — This is made of metal, and can be taken to pieces. It consists of a hollow cylinder, the floor of which can be elevated by a screw worked from below, so that by turning this the preparation is pushed upwards.

The vertical cylinder is fitted at its upper end with a horizontal steel plate. To cut sections with this instrument, the knife, a hollow-ground razor with a straight edge, is moistened with alcohol, and working on the plate, is drawn across it without using any pressure, so that the entire length of the blade is employed from heel to toe. A microtome suitable for ordinary purposes should have a cylinder with a diameter of 12 to 18 mm., and the distance between the edge of the plate and the central opening should be about 12 mm. In a microtome such as this any straight-edged razor may be used, while if the horizontal plate be larger or the cylinder wider, a blade of special construction will be required.

The cylinder microtome can only be used for cutting tissues previously hardened. If they can withstand a fair amount of compression without undergoing any important alteration they may be embedded in a piece of lardaceous liver or elder-pith. The cylinder opens in two halves, and between them the embedding material is fitted by paring, so as to be firmly held when the two halves are united.

With delicate tissues, as much melted paraffin as will cover the bottom of the cylinder is poured in. The piece is then placed in the cavity of the cylinder, and the space remaining round or above it is filled up with melted paraffin. The tissue should be surrounded with paraffin, but it is not necessary that it should be thoroughly saturated by it as in embedding.

If the tissues be placed for a few hours beforehand in some solvent of paraffin such as xylol, benzole, bergamot oil, etc., the paraffin will adhere to it better. The paraffin can afterwards be removed from the sections by the use of the same solvents.

Cylinder microtomes with a clamp for fastening them to

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the table are not convenient. Microtomes in which the cylinder cannot be taken to pieces, and those having the flat plate at the end covered with glass, are unsatisfactory.

2. The **sliding microtome** is a much more perfect instrument. By its use thinner, more uniform, and considerably larger sections can be obtained.

In this instrument the knife is carried on a slide, which moves up and down on three polished and well-oiled metal rails.

The tissue is either directly raised from below by a screw clamp, or, by means of a screw arrangement, can be slowly moved up an inclined plane, and thus progressively elevated a certain number of divisions along a graduated scale.

The tissue is either fixed directly into a clamp, or is held in a cylinder.

The various methods already mentioned (*vide* p. 27) for embedding the piece of tissue in lardaceous liver or in elder-pith may be employed, or it may be cemented on a piece of cork and fixed in the clamp.

The latter method is the one generally employed. A piece of the tissue with parallel surfaces is cut and fastened to a cork with glue. The upper surface of the cork has been previously notched with a knife. The cork with the tissue on it is then placed for some hours in absolute alcohol or strong spirit, to fix and harden the glue.

A mixture of glycerine and gelatine forms a cement which sets more rapidly and firmly. Gelatine is soaked in water for a few hours; the water is poured off, and the swollen gelatine boiled with an equal volume of glycerine, to which a little camphor, or better, a few drops of weak corrosive sublimate solution, have been added to prevent the formation of mould. The hot mixture is filtered through muslin and then solidifies.

In using this a small piece the size of a pea is melted over a flame on the point of an old knife. The drop of liquid is transferred to the cork, and the piece of tissue placed upon it. This is rapidly hardened in alcohol. Care must be taken, however, that the pieces which are to be fastened to the cork are not too thick. They should not be more than 8 mm. high, or otherwise they will be apt to bend under the knife.

The knife should be placed as obliquely as possible to the preparation, so that the entire length of the blade can be brought into play.

Only with very small and very hard pieces of tissue should the position of the knife be more nearly at right angles. These general rules do not apply to all cases; sometimes different positions of the knife must be tried until one is found which suits the preparation.

With the sliding microtome, the knife and the piece of tissue must both be kept constantly moistened with spirit, applied by a brush.

The sections can be readily removed with a curved blunt-pointed needle, which must not be allowed to touch the edge of the blade.

Tissues embedded in paraffin are cut dry, and are therefore apt to curl up. There are various devices (section stretchers) for preventing this; one of these being a piece of bent wire attached to the knife, the front part of which keeps the section flat. The simplest way of preventing the rolling up of paraffin sections is to hold a narrow strip of cardboard with the left hand on the knife, so that the sections are flattened on the blade. A small horn spatula can be used in a similar manner.

3. With **Gudden's microtome** sections can be cut under water. This is especially suitable for very large sections, as of the brain. The tissue to be cut is pushed up in a metal cylinder by a piston. The cylinder and the knife are in a vessel containing water. The tissue is fixed in the cylinder by surrounding it with a mixture of

Stearine	12 parts.
Lard	12 „
Wax	1 part.

4. The **Freezing microtome** is in very general use. The piece of tissue lies on the upper surface of a metal plate, on the under surface of which an ether spray plays. The pieces should not be more than 4 mm. high, otherwise the freezing is difficult and irregular. Before being frozen they should be thoroughly washed in water, and must not contain a trace of spirit. The complete removal of the spirit is more quickly effected in water raised to a temperature of 30° C. Tissues which have been hardened in spirit should remain as a rule for at least one night in a large bulk of water. Celloidin preparations which have been in 80 per cent alcohol should also be placed for at least twelve to twenty-four hours in a large volume of water. Pieces out of Müller's fluid may be cut by the freezing microtome at once, or after quite a short immersion in water, the

completion of the hardening process in alcohol before cutting being unnecessary.[1]

The piece of tissue should be lightly pressed on the metal plate with the handle of a scalpel until it is frozen. If the tissue does not become firmly frozen, it is probably because it has not been quite freed from alcohol, and it should then be replaced in water for a few hours. The freezing can often be accelerated by filling up any chinks which may exist between the tissue and the metal plate with a drop of water applied with the handle of a needle, or the under surface of the piece of tissue may be smeared with a little liquid gum.

The piece should be thoroughly frozen throughout, but not too hard, otherwise it becomes brittle and the sections will be striated.

The piece of tissue is so placed on the plate of microtome that the knife is brought to bear on an angle, and not on one of its sides.

The sections are removed from the blade of the knife into 80 per cent spirit. They will then unroll better later than they would if at once placed in water.

Fresh tissues are so altered by freezing that they should be cut by some other method. The freezing microtome is of great use, however, in cutting sections of tissues hardened in Müller's fluid, as they are unaltered by freezing, and lengthy processes, such as fixing on cork, etc., are obviated. There is a further advantage in that the sections thus obtained are thinner and more uniform than those obtained by most other methods.

Tissues hardened in alcohol and embedded in celloidin can be cut with very satisfactory results by the freezing microtome.

Microtomes may be obtained from

Jung, Heidelberg.
Katsch, Munich.
Schanze, Leipzig. } Sliding microtomes.
Becker, Gottingen.
Reichert, „

R. Kanthack, 21 Golden Square, W., is the agent for most of these firms.

Swift, 81 Tottenham Court Road, London, for Williams's freezing microtome.

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[1] The piece of tissue is often soaked in a mixture of gum (B.P. strength) 2 parts, syrup 1 part, for some hours before cutting. By this means the tissue, when firmly frozen, is less likely to splinter.—ED.

Cambridge Scientific Instrument Co. for the rocking microtome.
Cathcart's microtome (useful for cutting fresh tissues in the post-mortem room) can be obtained from Fraser, 22 Teviot Place, Edinburgh.

CUTTING IN SERIES.

Special methods of preparation are required for cutting sections in series.

(1) Celloidin Preparations. Weigert's Method.

Strips of tissue-paper are prepared so that their breadth is about double that of the sections to be subsequently placed upon them. The section directly it is cut is carefully drawn with a needle towards the edge of the blade, if not already there. The strip of paper is then placed on the upper surface of the section. They stick together, and are then moved to the left, parallel to the edge of the blade. To ensure the successful removal of the sections, the amount of alcohol used must be small. Each successive section takes its place to the right of the preceding one. The strips of paper must be kept moist, not only while the sections are being placed on them, but also when they have received their full number, and while further strips are being similarly treated. For this purpose a shallow dish is placed near the microtome, in which is put a single layer of blotting-paper soaked with alcohol covered over by a sheet of tissue-paper. The strips of paper are placed in it with the sections lying uppermost.

When the whole piece has been cut, each strip of paper, with the sections on its under surface, is gently pressed down upon a long glass slide covered with a film of collodion. The paper can then be withdrawn, leaving the sections fixed by the collodion in their proper order. Any free fluid should be removed, but the sections must not be allowed to dry. They are then covered over with a thin, even layer of collodion, and the sheet of glass is placed on its edge. The order of the sections is marked on it with a fine-pointed pencil dipped in methylene blue.

If the slides are now placed in a staining fluid (such as Weigert's hæmatoxylin solution for staining the central nervous system), the entire mass of collodion, with the sections enclosed in it, soon separates from the slide.

When the staining is completed the sheets of collodion are cut up under water with scissors as desired, and are then placed in 96 per cent (not absolute) alcohol. The sections should not be cleared with oil of cloves, nor in origanum oil, as they are not completely dehydrated, but should be passed through a mixture of xylol three parts, pure carbolic acid one part. On the bottom of the bottle containing this mixture, which may be used over and over again, is placed some white ignited sulphate of copper. When this turns blue it should be replaced by a fresh supply, or be re-ignited (*vide* p. 12).

(2) Serial Cutting by the Paraffin Method.

The sections are fixed in their proper order on the slides with a mixture of clove oil and collodion three to one. The further treatment, such as removal of the paraffin, staining, etc., has already been described (p. 24).

The slides should be numbered. If the tissue has not been previously stained in bulk, the sections may be stained on the slides. **Strasser** has devised a modification of Weigert's method, which is useful when the sections are so large that a complete series of them cannot be contained on one slide.

Sheets of paper, to take the place of Weigert's collodionised glass plates, are prepared as follows:—

A sheet of writing-paper is stretched out flat, and painted over with a mixture of four parts mucilage gummi acaciæ (official) and one part glycerine. When dry, collodion is painted over it as quickly as possible. The collodion should previously have been reduced to the consistency of glycerine by the addition of ether, and then one-hundredth part by volume of castor oil is mixed with it. Several coats of this mixture should be applied. Before cutting the paraffin preparation the sheets of paper which have been treated in this manner are painted over with an adhesive film composed of

Collodion . .	2	parts by volume.
Ether . . .	2	,, ,,
Castor oil . .	3	,, ,,

The sections are laid as evenly as possible in this cement, and then covered with more of the same mixture.

To remove the paraffin the sheets are placed for from half an hour to several hours in turpentine, and then in chloroform. They are then immersed in 80 to 85 per cent alcohol. Before staining they are placed in water or in 10 per cent alcohol, which dissolves the gum and separates the paper, so that the sections can then be treated as in Weigert's method (*vide* p. 36). The separation of the paper only takes place in aqueous staining solutions.

(3) **Darkschewitsch** has quite recently introduced a simple method for preparing and mounting sections in their proper order.

A glass cylinder or vessel, with a circumference approximately equal to that of the sections to be treated, is filled with spirit. Discs of blotting-paper, with a diameter rather less than that of the vessel, numbered with a black lead pencil, are laid in order and well soaked with spirit. Each section is removed from the blade of the knife by gently pressing one of these discs of blotting-paper upon it, and then withdrawing it. These discs of paper are then piled up in the cylinder, one above the other, the surfaces bearing the sections being uppermost, so that each section is now lying on the piece of paper bearing the corresponding number.

The sections may be kept like this for any length of time. To stain them, the spirit is poured off, they are washed with distilled water, and the staining solution poured upon them. Any other reagents may be used in the same way, or may be employed in shallow plates. Up to this point the sections have remained fixed to the discs of paper.

CHAPTER VIII

STAINING PROCESSES, ETC.

In the examination of unstained sections made from hardened tissues any of the methods may be employed which were given in Chapter II. on the examination of fresh tissues. They may be examined either in pure glycerine, or in glycerine diluted with an equal quantity of water.

Brushing, shaking, and artificial digestion have still to be mentioned as methods for bringing special elements into prominence. Brushing and shaking are the methods specially adapted for demonstrating the connective tissue framework in glandular organs, or for examining the stroma of tumours, such as carcinomata, which is effected by the removal of the cells.

Brushing is done in this way. The section is spread on a slide, and fixed on one side by a needle, whilst a fine brush is carefully and repeatedly passed over it from the needle to the opposite side, so that by degrees all the cells are removed. The brush may be used in the same manner in a dish filled with water.

In **Shaking**, the section is placed in a test tube partly filled with water, and shaken until the cells fall out.

The process of **Artificial digestion** has not hitherto been much employed in pathological work. The sections or pieces of tissue are exposed for several days to the action of trypsin solution in an incubator. They are then well shaken in a test tube, and examined in salt solution.

The trypsin solution is prepared as follows — A fresh ox-pancreas is treated with alcohol and ether in an extracting apparatus until a white friable mass is left. One part of this residue with five to ten parts of a 0·5 per cent salicylic acid solution is heated for three to four hours at 40° C., and is then filtered.

By the action of the trypsin the cells and cement substance of the tissue are removed, leaving the fibrous stroma.

General Methods of Staining. Treatment and Mounting of Stained Sections.

Modern methods of staining depend upon the fact that the various tissues exhibit different degrees of affinity for different stains, so that by the use of an appropriate colouring fluid a particular tissue may be made to stand out from the surrounding structures, and thus rendered distinct.

This affinity of certain tissues for particular colouring matters is often well shown in single staining, in which only one form of tissue may be fully stained, or to a much more marked extent than the rest. In other cases all the structures in a section appear equally stained at first, but after using some decolourising agent one only perhaps retains its colour.

The cell-protoplasm and the nucleus take up varying degrees of the same stain — a point of great histological importance. Nuclear-stains either stain the nucleus alone, or much more deeply than the surrounding protoplasm. The differentiation of tissues by a single stain may be carried still further by double staining. The two dyes are used either together or successively. Different structures or the constituents of cells (nucleus and protoplasm) are thereby picked out in different colours.

The following general rules apply to the use of staining agents:—

1. All staining fluids should be filtered before use. A small glass funnel with a filter paper may be conveniently kept in the mouth of each bottle of staining fluid. The filter paper should be renewed about every month.

2. The sections must be spread out as much as possible in the staining fluid, and must not be allowed to lie closely in contact with each other, otherwise very unequal staining will result. It is advisable on this account to cautiously move the sections about in the staining solution either with a needle or by gently blowing on the surface of the fluid. It is very important to employ sufficiently large dishes and a considerable quantity of staining fluid. This fluid may be poured back into the bottle each time after being used.

3. The time required for satisfactory staining varies within certain narrow limits. It is influenced not only by the time the staining fluid has been kept, as older solutions generally stain

more quickly and more deeply than those freshly prepared, but is also affected by the nature of the hardening and preserving processes which have been employed, and by the age of the tissue. Older preparations often stain more slowly and less deeply than recent ones. There is a considerable variation in the affinity possessed by the cells of different organs for colouring matters.

When a tissue stains with difficulty the process may often be aided as follows:—

a. By the prolonged action of the staining fluid up to twenty-four hours.
b. By employing a stronger solution of the staining fluid.
c. By warming the staining fluid, and especially by keeping the tissue in the fluid for a long time in an incubator.

4. The sections should be very carefully washed in distilled water until the water no longer becomes tinged.

It is advisable after washing the sections to leave them for several hours in a large volume of distilled water.

The sections are then, with some exceptions, mounted and examined in either **glycerine** or **Canada balsam.** Dammar varnish is generally unsuitable as a mounting medium, especially for hæmatoxylin preparations. The sections may be transferred to glycerine directly from distilled water. Hæmatoxylin preparations should however be washed in water for a long time before they are mounted in glycerine. The lifter is used for transferring the sections, and any superfluous water is then removed with blotting-paper.[1] It is often most convenient to place the sections in water on the slide, to remove the water with blotting-paper, and then to add the glycerine. The necessary quantity of glycerine can be then more easily estimated.

Air must be excluded from sections mounted in **glycerine.** For this purpose the edge of the cover-glass is smeared with wax by drawing round it the wick of a wax candle which has just been extinguished. This leaves behind it a narrow streak of melted wax, which solidifies at once. If the cover-glass slips about during this process the four corners should first be fixed by a drop of wax from the wick.

It is essential in edging with wax that there should not be any excess of glycerine oozing out from under the edge of the cover-glass. Should this be the case it must be wiped away with a linen rag dipped in absolute alcohol. Any glycerine which oozes out at any point while the wax is being applied must be removed.

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[1] Or the section may be spread out on the slide by introducing the slide obliquely below the surface of the water under the floating section. One corner of the section is fixed by a needle and the slide gradually raised.—Ed.

If the specimen is to be kept for any length of time, the edging of wax is covered over with cement or with gold size. This should completely cover the layer of wax so as to come directly in contact with both the slide and the cover-glass. The layer of wax should therefore be quite narrow. The varnish should not be applied directly to the cover-glass without the previous coating of wax, because the varnish remains fluid for some time, and is liable to be drawn under the cover-glass by capillary action.

Several kinds of varnish are used for edging specimens, but asphalte and gold size are sufficient for ordinary purposes, as pathological sections are seldom mounted in glycerine.[1]

Sections stained with aniline dyes should not be mounted in glycerine, as this gradually dissolves out the colour. Sections stained with Bismarck brown or with some special (aniline) lardaceous reagent are the only exceptions to this rule.

Sections mounted in **Potassium acetate** (saturated solution) may be edged round in the same way. Potassium acetate is particularly adapted for tissues stained with aniline colours in cases where Canada balsam, which is otherwise always preferable, cannot be employed.[2]

Canada balsam, dissolved in xylol, is the mounting medium most generally employed in pathological histology.

The sections, however, must first be thoroughly dehydrated, or else they will become clouded in the Canada balsam.

In this process the sections are transferred from water to 96 per cent spirit for three to five minutes, and then for the same length of time to absolute alcohol, the volume of which must naturally depend on the number of sections to be treated. As sections which have been in water are apt to crinkle when placed in strong spirit, they should be removed from the distilled water by means of a small piece of filter paper pushed under them, and thus lifted into the spirit. It is less tedious to transfer them with a proper lifter. The lifter is drawn away from under the section in the alcohol as soon as it has begun to get rigid. The crinkling of the sections may be also obviated by placing them first in 96 per cent, and after this in absolute alcohol, where they should be at once spread out with a needle. This is decidedly preferable to using absolute alcohol from the first and then renewing it.

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[1] Hollis' liquid glue will be found useful.—ED.

[2] *Farrants' solution* may be used for mounting sections out of water, and is particularly adapted for specimens stained with picro-carmine, which should not be washed before they are mounted. Sections mounted in Farrants' solution require ringing round, as it does not set.—ED.

Absolute alcohol does not combine well directly with Canada balsam dissolved in xylol, so on this account the sections are now placed for one to three minutes in some fluid which is capable of mixing both with the alcohol and with the Canada balsam, and which has in addition the very important property of rendering the specimens clearer and more transparent.

These properties are possessed by several of the ethereal oils. **Turpentine** is now but rarely used. It gives a fine colour to tissues injected with Berlin blue.

Oil of cloves is very generally employed. It has the advantage of not being affected by slight traces of water still retained in the sections. On the other hand, it cannot be used with celloidin preparations, as it dissolves it at once. In addition, it causes aniline colours to fade, or gives them a dirty dull colour.

Oil of bergamot clears well, and may be used with celloidin preparations. Likewise **oil of marjoram** (origanum oil), **cedar-wood oil,** and **lavender oil.** **Xylol,** to which pure carbolic acid (*vide* p. 37) has been added, is excellently suited for sections stained with aniline colours, especially for those containing micro-organisms. The sections are, however, apt to crinkle in this fluid.[1]

It is usually sufficient to leave the sections in the clearing reagent for two to three minutes. Sections which are somewhat thick may be rendered so transparent by remaining longer (a half to several hours) in it that they can afterwards be examined quite well under high powers.

A section may be cleared by spreading it out in a drop of the oil on a slide. If many sections are to be mounted, it is better to transfer them from the alcohol to a dish containing the oil, and to remove them to the slides by a lifter.

The excess of oil is removed with blotting-paper, and lastly, the section is covered with a drop of Canada balsam, and a cover-glass lowered on to it. Should the Canada balsam be thick, it can be made to spread more evenly by gently warming the under surface of the slide over a spirit lamp.

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[1] Mineral or *coal naphtha* also should be mentioned. It has many advantages over xylol for embedding in paraffin, and for clearing after aniline dyes. It is about one-sixth the price, renders the tissue less brittle, and does not cause so much shrinking; it does not evaporate so quickly, and has a greater power of penetration.—ED.

If the Canada balsam is too liquid the sections should be left for some days lying exposed to the air, but protected from dust, so as to harden it.

The various processes of staining and mounting may be summarised as follows :—

1. Stain.
2. Wash in distilled water.
3. Transfer on a lifter to 96 per cent alcohol for three to five minutes.
4. Transfer on a lifter to absolute alcohol for three to five minutes.
5. Transfer to the clearing fluid.
6. Spread out on the slide.
7. Remove the excess of oil with blotting-paper.
8. Canada balsam, cover with the cover-glass.
9. Slightly warm the slide.

NUCLEAR-STAINING.

Alum Hæmatoxylin.

Preparation: Crystals of commercial hæmatoxylin are dissolved in a small quantity of absolute alcohol. The solution should be quite concentrated and contain crystals in excess. To this a 1 per cent aqueous solution of alum is added until the whole becomes a light blue or violet colour. It is then exposed to light, and in a few days acquires a deep blue colour, and is then ready for use. As a guide to the amount of the alcoholic solution of hæmatoxylin which must be added, it may be borne in mind that a satisfactory staining solution usually contains from $\frac{1}{2}$ to $\frac{3}{4}$ per cent of pure hæmatoxylin. As the solution stains more deeply the older it gets, it is often advisable after a few weeks to dilute it further with alum solution.

It is used as follows :—

(1) Stain for two or three minutes.
(2) Wash in plenty of distilled water.
(3) Leave the sections in distilled water for twelve to twenty-four hours.
(4) Alcohol, oil, Canada balsam.

Hæmatoxylin is one of the best and most certain nuclear stains, and can be used for most tissues.

The nuclei acquire a deep blue or violet colour, while the protoplasm of the cells is stained a fainter though similar tint.

In fresh hæmatoxylin solutions sections stain slowly, but allowance must always be made for the fact that they become darker afterwards in water. When the solution is fairly old they generally stain in one, or even half a minute. It is therefore advisable to ascertain the progress of the staining by examining the sections at short intervals in distilled water. A trial section may be examined at once in glycerine after washing, or dehydrated and mounted in clove oil. After careful washing the sections should be left in water for at least twelve hours. The colour is fixed by the dehydrating process, but sections often grow darker even after mounting in Canada balsam.

Sections overstained by the use of old solutions of hæmatoxylin may be partially decolourised by putting them back again for several hours in a 1 per cent aqueous solution of alum. After this the sections must remain in distilled water for at least twelve hours. Sections stained with hæmatoxylin should on no account be brought into contact with acids.

After keeping for six months, solutions of alum hæmatoxylin turn a reddish colour, and should then be discarded and a fresh solution prepared.[1]

Ehrlich's Acid-Hæmatoxylin.

Preparation :	Hæmatoxylin .	2·0 grms.
	Absolute alcohol	60·0 ccm.

Add to this—

Glycerine .	60·0 ccm.	Saturated with alum.
Distilled water	60·0 ,,	
Glacial acetic acid	3·0 ,,	

---

[1] *Hæmatein.* This substance is obtained as a brown powder from hæmatoxylin. It is a nuclear stain at least as good, if not better than hæmatoxylin. The solution is made as follows :—

(*a*)	Hæmatein . . .	1 grm.
	90 per cent alcohol . .	50 ccm.
	Dissolve by heating.	
(*b*)	Alum	50 grms.
	Distilled water . .	1000 ccm.

Mix (*a*) and (*b*). Allow to settle, and use the clear fluid for staining. If too strong, dilute with water or alum solution. After staining wash in 1 per cent alum solution. It is a very useful dye for staining in bulk.—Ed.

It is used as follows :—

(1) Stain for three to five minutes.
(2) Wash in water.
(3) Alcohol, oil, Canada balsam.

After preparation, Ehrlich's hæmatoxylin solution should be exposed to the light for two to three weeks and afterwards filtered. It does not readily overstain, and keeps very well. It has no advantage over alum hæmatoxylin solution, which is prepared in a simpler manner, and defines the nuclei more sharply.

Heidenhain's Method of Staining with Hæmatoxylin.

(1) Stain for twenty-four to forty-eight hours in a simple 0·5 per cent aqueous solution of hæmatoxylin (prepared by warming).

(2) Place the sections for twenty-four to forty-eight hours in a 0·3 per cent solution of potassium monochromate, which should be repeatedly changed as long as the liquid becomes tinged.

(3) Wash very thoroughly in water.

(4) Alcohol, oil, Canada balsam.

This method gives very sharp definition, and is specially suitable for staining tissues in bulk. After washing in water the pieces of tissue are then hardened in alcohol of successively increasing degrees of strength, and are then embedded. They acquire a very dark colour, and on this account the sections cut should be thin.

Apathy has devised a modification of Heidenhain's method by which the prolonged immersion of the tissue in the aqueous solution is avoided. A 0·5 per cent alcoholic solution of hæmatoxylin, and also an alcoholic solution of potassium bichromate, are used. The latter is prepared by adding two volumes of absolute alcohol to one of a 0·5 per cent aqueous solution of potassium bichromate.

In other respects the method is the same as Heidenhain's.

Alum Carmine.

Preparation : 2 to 5 grms. of carmine are boiled for a half to one hour with 100 ccm. of a 5 per cent alum solution, cooled and filtered.

It is used as follows :—

(1) Stain from ten minutes to two hours.
(2) Wash in water.
(3) Alcohol, oil, Canada balsam.

The nuclei are stained a beautiful violet-red colour. An advantage which it possesses as compared with hæmatoxylin is that no overstaining takes place even when the sections are left in it for several hours. The sections do not require so much washing in water as is the case with hæmatoxylin, and the colour is not so sensitive to acids. On the other hand, it is not suitable for tissues which stain with difficulty.

Lithium Carmine.

Preparation :	Carmine . . .	2·5 to 5·0 grms.
	Saturated aqueous solution of lithium carbonate .	100· ccm.

It is used as follows :—

(1) Stain for two to three minutes.
(2) Wash for a half to one minute in acid-alcohol (*i.e.* strong hydrochloric acid 1·0 ccm. to 70 per cent alcohol 100·0 ccm.).
(3) Wash in plenty of distilled water until free from acid.
(4) Alcohol, oil, Canada balsam.

Lithium carmine stains nuclei a deep red colour. It is a very sure nuclear stain, and acts rapidly and well even with tissues which do not stain easily.

In the case of tissues which stain with difficulty the strength of the solution may be increased up to 5 per cent. The simplicity of its preparation is a further advantage. Overstaining can be easily avoided by prolonged washing in acid-alcohol, which removes any excess of colour. On the other hand, the fact that hydrochloric acid is used with this stain must always be taken into account (*vide* p. 45). Lithium carmine is well adapted for tissues injected with blue colouring mass.

Borax Carmine.

Preparation :	Carmine . .	0·5 grm.
	Borax . .	2·0 „
	Distilled water .	100·0 ccm.

These are well mixed and heated to boiling; while continually stirring it add

Acid. acetic. dil. (B.P.) 4·5 ccm.

Allow it to stand for twenty-four hours and then filter.

It is used as follows:—

(1) Stain from five to fifteen minutes.
(2) Wash for a half to one minute in acid alcohol (hydrochloric acid 1 ccm., alcohol (70 per cent) 100 ccm.
(3) Completely remove the acid by washing in water.
(4) Alcohol, oil, Canada balsam.

Borax carmine stains like lithium carmine, but the colour is not so deep.

Picro-Carmine.

Preparation:		
	Carmine . .	1·0 grm.
	Liq. ammoniæ .	5·0 ccm.
	Distilled water .	50·0 ,,

To this add

A saturated aqueous solution of picric acid 50·0 ccm. The staining fluid is allowed to stand in a wide, uncovered vessel until the ammonia has completely evaporated, and is then filtered.

It is used as follows:—

(1) Stain for one hour.
(2) Wash for half an hour in glycerine containing 1 per cent hydrochloric acid, which should be coloured slightly yellow by picric acid.
(3) Wash for five minutes in water, which should be also slightly yellow by the addition of picric acid.
(4) Dehydrate in alcohol, which is also coloured with picric acid.
(5) Oil, Canada balsam.

Picro-carmine has this advantage, that it produces a permanent double-stain if employed in the manner described, and when a small quantity of picric acid has been added to the glycerine, water, and alcohol. The nuclei become a brownish-red colour while the protoplasm is stained yellow. It has also the further advantage that tissues which have undergone hyaline or colloid degeneration acquire a deep yellow colour. The protoplasm of striated muscle and the corneous layer of the skin are clearly differentiated by their marked yellow colour.[1]

Weigert's picro-carmine solution as obtained from Grübler in Leipzig is a very reliable stain.[2]

Picro-lithium Carmine.

Preparation: To the lithium carmine solution described above (p. 47) are added two parts of a saturated aqueous solution of picric acid.

It is employed in the same way as picro-carmine, and stains in a similar manner.

Beale's Carmine.

Preparation:	Carmine	0·6 grm.
	Liquor ammoniæ	3·75 ccm.

Boil this for a few minutes and then add:—

Glycerine	60·0 ccm.
Distilled water	60·0 „
Alcohol	15·0 „

It is used as follows:—

Beale's carmine solution is mostly employed for staining in bulk. For this purpose the pieces must remain in the solution for two to eight days, according to their thickness. They are afterwards washed in water, hardened in alcohol, and embedded.

Bismarck Brown.

This is prepared either as—

(*a*) A 3 to 4 per cent saturated aqueous solution, which is boiled and filtered,

or

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[1] Sections which have been stained in picro-carmine may be mounted directly in Farrants' solution (*vide* footnote, p. 42), or the unstained section may be placed on a slide, a drop of picro-carmine added, and gently warmed over a spirit-lamp. The excess, but not the whole of the stain, is removed with blotting-paper, and the section mounted in Farrants' solution. The advantage of this is that the staining process continues for a day or two. Picro-carmine is perhaps the best general stain for sections of fresh tissues. Sections mounted in Farrants' solution must be edged round (*vide* footnote, p. 42).—Ed.

[2] Excellent picro-carmine can be obtained from Frazer, 22 Teviot Place, Edinburgh.—Ed.

(*b*) A concentrated alcoholic solution, 2 to 2½ per cent in 40 per cent alcohol.

It is used as follows:—

(1) Stain for five minutes.
(2) Wash in strong spirit.
(3) Alcohol, oil, Canada balsam.

Bismarck brown stains nuclei a beautiful brown colour. Bacteria, when present, are stained deep brown. The protoplasm acquires a light brown tint. The staining is the same with both aqueous and alcoholic solutions. There is no danger of overstaining. Sections thus stained are especially suitable for micro-photography. The sections may be washed in 1 per cent acid-alcohol instead of strong spirit (*vide* p. 47).

Only a few aniline colours besides Bismarck brown are usually employed as nuclear-stains, although most of them may be used in the same manner. Unless special precautions are adopted, they stain the tissues diffusely. The differentiation of the nucleus can only be produced by washing in absolute alcohol. The colour is not so permanent as that produced by hæmatoxylin and carmine solutions.

Of the aniline dyes used as nuclear-stains, gentian violet and Biondi-Heidenhain's stain, which is a modification of Ehrlich's stain for blood (*vide* p. 118), may be used. The latter is, however, not permanent.

Gentian Violet.

A 1 per cent aqueous, or a 1 to 2 per cent alcoholic solution is employed.

It is used as follows:—

(1) Stain for three to five minutes.
(2) Wash in alcohol until the sections acquire a light-blue colour.
(3) Absolute alcohol, xylol, Canada balsam.

The nucleus may be further differentiated by placing the stained section in a 1 per cent aqueous solution of acetic acid for not more than half a minute. After this it is washed in alcohol.

Biondi-Heidenhain's Stain (Ehrlich-Biondi).

Preparation:

A filtered saturated aqueous solution of aurantia	100 ccm.
A saturated solution of acid fuchsine . .	20 „
Methyl-green, saturated solution . .	50 „

To prepare these saturated solutions they must be allowed to stand for several days with an excess of the various colouring matter.

For staining, this solution is diluted with 100 parts of water, and acetic acid added until it becomes a decidedly deeper red colour. A drop placed on a piece of blotting-paper should appear bluish-green in the centre and orange at the edges. If the orange-coloured zone is surrounded by a red band the solution contains an excess of fuchsine. Old solutions which have lost some of their staining power may again be rendered fit for use by adding a very small quantity of acetic acid. A glass rod dipped in acetic acid is swung to and fro in the air, and then dipped into the staining solution.

The solution prepared by Heidenhain's method can be obtained from Dr. Grübler of Leipzig.

It is used as follows:—

(1) Harden the tissue in corrosive sublimate (*vide* p. 14).
(2) Stain for six to twenty-four hours in the dilute solution.[1]
(3) Wash for a short time in 90 per cent alcohol.
(4) Dehydrate in absolute alcohol.
(5) Xylol, Canada balsam.

Nuclei undergoing karyokinesis and the fragmentary nuclei of leucocytes stain an intense green violet, while nuclei at rest are coloured blue, and the red blood corpuscles red.

The method is particularly useful for preparations containing many leucocytes. It was originally only intended for paraffin sections and for staining on the slide. Single sections in celloidin may, however, be stained in the solution without previously fixing them on a slide. The celloidin need not be removed beforehand if the sections are very thin. In thicker sections, however, its presence is a disadvantage, as it stains irregularly.

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[1] By Dr. Ruffer's courtesy I am enabled to state that better results are obtained if the sections be dipped in water between stages two and three.—Ed.

Nikiforoff very strongly recommends for glandular organs Ehrlich's stain for eosinophile cells.

Aurantia Eosine Indulin	partes æquales 2·0 grms.
Glycerine	30·0 ccm.

The tissue is hardened in corrosive sublimate, and embedded in paraffin. The sections are stained on the slide.

DIFFUSE AND DOUBLE-STAINING.

Eosine and carmine neutralised with ammonia or neutral carmine, stain the ground substance of tissues, and are very largely used. Eosine may be employed either as an aqueous or as an alcoholic solution. It is far more often employed for double-staining in conjunction with some nuclear stain, such as hæmatoxylin, than as a single diffuse stain.

Neutral Carmine.

Preparation: 5 grms. of carmine in powder are rubbed up in a mortar with an excess of liquor ammoniæ so as to form a red paste, and this is then boiled with 200 ccm. of water in an open retort until the ammonia is driven off.

The retort is left open for some weeks until a red deposit settles. It is then filtered. The fluid stains better the older it gets, and on this account, after being used, should be filtered and put back again into the bottle.

The staining fluid may be prepared ready for immediate use by **Honegger's** method. Powdered carmine is mixed with just enough ammonia to produce a thick paste. This is then plastered thinly on the sides of the mortar and left to dry. When dry the mass is finely powdered, exposed to the air for twenty-four hours, and dissolved in cold water.

It is used as follows:—

A light red solution is prepared by adding some of the concentrated solution to water. The sections are left in this until they acquire a distinct red colour. It is best to leave the sections in a very dilute solution for a long time, up to twelve hours. If greater rapidity is required a stronger solution may be used, in which the sections become sufficiently stained in twenty to thirty minutes.

Afterwards they should be thoroughly washed in water.

In double-staining with hæmatoxylin and carmine the sections are always stained first with hæmatoxylin and then with carmine. After staining in hæmatoxylin they must be washed in water for at least six, preferably for twelve, hours. After this has been done they are stained in carmine, and again carefully washed in water.[1]

Eosine. (A) Aqueous Solution.

Preparation: A strong aqueous or alcoholic solution of eosine is added to water until it acquires a pink colour, and contains about one part of eosine in 1000 to 1500 of water.

It is used as follows:—

(1) Stain for a few minutes until the sections become pink.
(2) Wash in water.
(3) Dehydrate in alcohol, but not longer than is necessary, as the alcohol gradually dissolves out the eosine.
(4) Oil (not bergamot oil), Canada balsam.

Eosine. (B) Alcoholic Solution.

Preparation: A concentrated alcoholic solution of eosine is added drop by drop to absolute alcohol until it acquires a pink colour.

It is used as follows:—

(1) The sections, which should previously have been placed in 96 per cent alcohol, are left in the stain from a few minutes up to several hours.
(2) Wash in alcohol until the desired tint is obtained.
(3) Oil, Canada balsam.

The time required for staining with eosine varies so considerably that no fixed limits can be given; sometimes as long as twenty-four hours may be required.

Alcoholic solutions of eosine stain more uniformly than aqueous solutions. Eosine stains red blood corpuscles a pink to a copper colour, and is therefore a good selective stain for blood-vessels.

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[1] *Vide* footnote, p. 14.

The tissues acquire a diffuse pink colour. On this account eosine is seldom used alone, but is usually employed in combination with hæmatoxylin, alum carmine, or gentian violet. In this method of double-staining the nuclear stain must be first employed and then the eosine. A few drops of an alcoholic solution of eosine are added to the absolute alcohol, through which the sections stained with hæmatoxylin are being passed, after which the sections are washed in fresh absolute alcohol. Or again, eosine may be added to the oil of cloves or origanum oil, so as to stain the sections during the clearing process. Oil of cloves, in which a large number of eosine preparations have been cleared, often takes up the colour to an undesirable extent, and may therefore tinge other sections subsequently placed in it.

Eosine may also be added to the hæmatoxylin solution, so that a double stain is produced by the one staining fluid. This may be done by adding 0·5 grm. of eosine to Ehrlich's hæmatoxylin solution (*vide* p. 45). It does not, however, give better results than staining separately in hæmatoxylin and eosine.

As a diffuse stain for tissues **picric acid** is used in picro-carmine (*vide* p. 48) and in picro-lithium carmine (p. 49).

There is no advantage in dividing this process into two stages: (1) lithium carmine; (2) picric acid 1 to 5 per cent. They can be combined in one process. A satisfactory double stain may be obtained by alum carmine and picric acid used separately.

Van Giesson's Method.

(1) Harden in Müller's fluid or in alcohol.
(2) Stain for three to ten minutes in hæmatoxylin.
(3) Wash thoroughly in water.
(4) Stain for three to five minutes in a saturated aqueous solution of picric acid, to which a saturated aqueous solution of acid-fuchsine is added, until the mixture acquires a deep red colour.
(5) Wash in water for half a minute.
(6) Spirit, absolute alcohol, origanum oil, Canada balsam.

This method is very convenient, and gives a beautiful double stain. The nuclei are brown-red, the interstitial substance bright red.

Lardaceous, colloid, hyaline, and mucoid material are also stained by this method. Van Giesson originally recommended Delafield's hæmatoxylin, but ordinary alum hæmatoxylin gives very good results. The sections should be overstained, because the picric acid decolourises them to a certain extent.

STAINING IN BULK.

This is a special method in which the piece of tissue is stained as a whole before being cut into sections. The staining solutions should contain a certain amount of alcohol, such as Beale's carmine (p. 49), Heidenhain's hæmatoxylin (p. 46), and Bismarck brown (p. 49).

In staining in bulk the following precautions are necessary :—

(1) The hardened or "fixed" piece of tissue should not be too large, or else the staining solution penetrates very slowly.
(2) The piece of tissue must be left in the stain much longer than is the case with sections, viz. from one to three, three to four, four to eight days.
(3) After staining, the tissue should be very thoroughly washed until no more colour comes out.
(4) Harden the tissue in 96 per cent spirit or absolute alcohol.

The advantages of staining tissues in bulk are: that it is an economy of time, as it is quicker than staining after cutting; smaller quantities of reagents are used, and the amount of manipulation required is much diminished.

The method is, however, used much less in pathological than in normal histology, and the limits of its application diminish with the increasing introduction of special colour reactions corresponding to definite tissue changes.

As a rule, pathological tissues often require more than one single stain. In addition to the ordinary histological stains, methods for demonstrating bacteria may be necessary. Besides the ordinary stains some special reaction for degenerative changes, such as fatty or lardaceous, etc., may be required. But even in a purely histological examination it is in many cases impossible to decide beforehand which staining method will best suit the case. The more rapid manipulation of sections obtained from tissues stained in bulk loses much of its importance when compared with the results obtained from satisfactory celloidin embedding.

STAINING KARYOKINETIC FIGURES.

Special methods for fixing and staining are required.

The first essential is that the pieces to be examined must be removed immediately after the death of the animal, and placed at once in the fixing fluid. Not more than half an hour at most should have elapsed either after the death of the animal or after the removal of the tissue from the living body.

If this be not done decomposition changes set in, and the nuclear figures disappear.

Karyokinetic figures may, however, be sometimes made out when the fixing process has been delayed for a considerable time after death, but the details are neither so numerous nor so distinct as in tissues freshly fixed.

It is evident, therefore, that the fixing solution should permeate rapidly the whole tissue. To facilitate this the tissue should be cut into very thin pieces not more than 4 mm. thick before placing it in the fluid. This is the more necessary as most fixing fluids penetrate slowly.

It is seldom possible to demonstrate satisfactorily karyokinesis in tissues from the post-mortem room, as their removal is necessarily delayed for many hours after death. The method is more adapted to tissues removed from the living body by operation.

Karyokinesis may be demonstrated in tissues which have been hardened in alcohol or Müller's fluid (*vide* p. 13), but they must be placed in the hardening fluid in very thin slices, and when quite fresh.

There are many other fixing fluids better adapted for this purpose.

The piece of tissue should be placed in several times its bulk of the fixing solution, which should be renewed whenever it becomes at all turbid.

Flemming's solution is the best for this purpose.

Preparation:	2 per cent solution of osmic acid	4 parts.
	1 per cent solution of chromic acid	15 „
	Glacial acetic acid . .	1 „

If the Flemming's solution is employed for showing the presence of fatty changes, and not for karyokinetic figures, it is generally advisable to add a smaller quantity of acetic acid (0·3 to 0·5), so that the protoplasm may not be rendered too transparent.

It is used as follows:—

(1) Leave the pieces in the solution for one to three days.

(2) Wash in water for three to six hours.

(3) Harden successively in 30, 60, and 96 per cent alcohol, leaving the tissues in each for twenty-four hours.

(4) Embed in celloidin in the usual way. The sections must then be stained with saffranine.

Staining with Saffranine.

(1) Stain the sections for a half to twenty-four hours in a 1 per cent aqueous solution of saffranine.

(2) Wash in water for a very short time.

(3) Wash in absolute alcohol which has been very slightly acidified by the addition of a few drops of acid-alcohol (1 per cent). To a dish of medium size containing alcohol five to ten drops of acid-alcohol (*vide* p. 47) are added.

(4) Wash in absolute alcohol until the sections acquire a light reddish-brown colour.

(5) Oil, Canada balsam.

Flemming's method with subsequent saffranine staining has the advantage that resting nuclei are stained a pale colour, while dividing nuclei are much more deeply stained.

The position and approximate number of the dividing nuclei may by these methods be determined rapidly and with fair accuracy even under a low power.

Hardly any other stains differentiate resting from dividing nuclei.

Babes recommends aniline-water saffranine, which stains much more rapidly. Two parts of aniline oil are mixed with 100 parts of water, and an excess of powdered saffranine is added. Warm to 60° C. Filter. The solution will keep for two months. If for some reason it is not thought desirable to use saffranine, an aqueous solution of gentian violet or carbolfuchsin give very good results. A very useful modification of Flemming's mixture, described by **Hermann**, consists in replacing the chromic acid by a 1 per cent chloride of platinum solution. By this method the cell spindle, the central and polar bodies are shown very distinctly.

Sections of tissues which have been fixed in Flemming's solution may be stained in hæmatoxylin by Benda's method. This is a modification of Weigert's method for staining medullated nerve-fibres.

Benda's Hæmatoxylin Method.

(1) Transfer the sections to a concentrated solution of acetate of copper, and keep them in this for twenty-four hours in an incubator.
(2) Wash thoroughly.
(3) Stain in a 1 per cent aqueous solution of hæmatoxylin until the sections are black.
(4) Decolourise in hydrochloric acid, 1·500, until the sections turn yellow.
(5) Neutralise the acid in a saturated acetate of copper solution.
(6) Wash.
(7) Alcohol, oil, Canada balsam.

Corrosive Sublimate.

Tissues preserved in a saturated aqueous solution of corrosive sublimate (*cf.* p. 14) may also be treated so as to exhibit karyokinetic figures. Heidenhain-Biondi's method (*vide* p. 51) is very suitable for staining them. The resting nuclei are stained violet and the dividing nuclei green.

Chromic Acid Solution.

The strength employed is 0·3 to 1·0 per cent. It requires on an average from twenty-four hours to several days to act. Chromic acid penetrates with great difficulty, so the pieces of tissue should be particularly small.

Rabl's Chrom-Formic Acid.

Preparation: Four to five drops of concentrated formic acid are added to 200 cc. of a 0·3 per cent chromic acid solution. It takes twelve to twenty-four hours to act. Wash thoroughly. Harden in 30, 60, 90 per cent alcohol successively.

Fol's Fixing Fluid.

1 per cent	osmic acid	2	parts by	volume.
1 „	chromic acid	25	„	„
2 „	acetic acid	5	„	„
Water		68	„	„

Chrom-Picric Acid.

Concentrated aqueous solution of picric acid	10	parts by	volume.
1 per cent chromic acid . .	25	„	„
Water	65	„	„

Altmann's Fixing Method.[1]

(1) Leave in 3 per cent nitric acid for one hour.
(2) Wash thoroughly.
(3) Harden in alcohol.

Alcohol.

Alcohol alone acts as a fixing agent if the pieces of tissue are very thin. The process is facilitated and shortened by warming the alcohol. Sections may be stained by Bizzozero-Vassale's method.

Bizzozero-Vassale's Method.

(1) Stain for ten minutes in Ehrlich's gentian violet solution:—

Gentian violet .	1·0	grm.
Alcohol . .	15·0	ccm.
Aniline oil .	3·0	„
Water . .	80·0	„

(2) Wash rapidly in absolute alcohol.
(3) Transfer for two minutes to a solution of iodine in potassium iodide (Iodine, 1 ; KI, 2 ; water, 200).
(4) Thirty seconds in absolute alcohol.
(5) Thirty to forty seconds in chromic acid, 0·1 per cent.

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[1] In addition to the foregoing, the following will be found an excellent fixing solution : **Foa's Solution.**—Müller's fluid saturated with corrosive sublimate.—Ed.

(6) Twenty to thirty seconds in absolute alcohol.
(7) Thirty seconds in chromic acid, 0·1 per cent.
(8) Thirty seconds in absolute alcohol.
(9) Oil of cloves. This should be repeatedly changed until it no longer becomes coloured.

This method is a complicated one, and the large quantity of clove oil employed is a further disadvantage.

Baumgarten's Method.

This method for demonstrating karyokinesis may be used in combination with staining for bacteria.

(1) Harden for several weeks in a dilute chromic acid solution.
(2) Stain for five to ten minutes in a concentrated alcoholic solution of fuchsine.
(3) Wash in absolute alcohol for a short time.
(4) Stain for five to ten minutes in an aqueous solution of methylene blue. Methylene blue is used after the fuchsine to displace it from the interstitial substance, so that the karyokinetic figures become prominently stained.

To examine at the same time for micro-organisms, particularly for tubercle bacilli, the sections must be stained beforehand for twenty-four hours in an aniline-water solution of methyl-violet, and then decolourised with dilute acid. For staining other micro-organisms the treatment with acid is omitted.

Gram's Method (*vide* p. 80).

Gram's method for staining micro-organisms may also be used for karyokinesis. In tissues which have been cut into thin slices and hardened in alcohol, the resting nuclei are decolourised (often only partially) by Gram's method, while nuclei undergoing division retain their colour.

DEMONSTRATION OF "CELL INCLUSIONS"

Altmann's Method for Showing the Granules in Cells.

(1) Place the pieces of the tissues removed from the

recently-killed animal for twenty-four hours in a mixture of

5 per cent solution of potassium bichromate. } āā.
2 per cent solution of perosmic acid.

(2) Wash for several hours in running water.
(3) Harden in 75 per cent, then 90 per cent, and lastly in absolute alcohol.
(4) Saturate with xylol and embed in paraffin (*vide* p. 24).
(5) Cut.
(6) Remove the paraffin with xylol from the section which is fixed to the slide, then wash with alcohol.
(7) Place on it a few drops of a 20 per cent solution of acid-fuchsine in cold saturated aniline water. Warm over a flame until the fluid steams.
(8) Allow it to cool, then wash away the staining fluid with a mixture of a concentrated alcoholic solution of picric acid one part, with water two parts.
(9) Pour on it some picric acid solution, and carefully warm for thirty to sixty seconds on the paraffin oven.
(10) Pass it through alcohol and then xylol.
(11) Xylol, Canada balsam.

William Russell has described the following method for showing bodies occurring in the cells of tumours:—

(1) Harden in alcohol or Müller's fluid.
(2) Stain for ten minutes or longer in a saturated solution of fuchsine in a 2 per cent aqueous solution of carbolic acid.
(3) Wash in water for a few minutes.
(4) Wash in absolute alcohol for half a minute.
(5) Stain for five minutes in a 1 per cent solution of iodine green in a 2 per cent carbolic solution.
(6) Dehydrate quickly with absolute alcohol, oil of cloves, Canada balsam.

[*Note.*—In preparations which may require to be photographed under the microscope, the use of methylene blue should be avoided, as it is the worst stain which can be employed for this purpose.—Ed.]

CHAPTER IX

THE EXAMINATION OF DEGENERATED TISSUES

(A) NECROSIS.

Areas of necrosed tissue are best examined in the fresh condition in cases where they can be distinguished with the naked eye and isolated. The necrosed tissue is scraped with a knife, and the mass clinging to the blade is divided up in water or salt solution, or small portions are cut out and teased up in these fluids. This mode of examination is adapted for necrotic patches, such as occur in the brain, liver, heart, muscles, and lungs.

Necrosis in individual cells or in collections of cells must be examined in sections. Alcohol and Müller's fluid are the best hardening agents. It is generally advisable to embed the tissues in celloidin, otherwise parts of the necrotic patches may fall out. The usual nuclear dyes may be employed, viz. hæmatoxylin, alum carmine, lithium carmine. Parts of the tissue in which the cells and their nuclei have undergone necrosis no longer stain by nuclear dyes, and hence are distinguishable by their pale colour from the healthy and more deeply stained portions. In necrosed tissue, however, the staining produced is often diffuse and undifferentiated. Nuclei of irregular shape and unequal size may often be seen within the necrosed area which stained very deeply, and which may be regarded in part as the remains of destroyed nuclei.

Necrosed tissues stain diffusely with carmine or eosine. Weigert's fibrin stain (*vide* p. 121) is useful for distinguishing them from simple coagulation products, such as fibrin, as it gives a negative result in necrosis.

(B) SIMPLE AND PIGMENTARY ATROPHY.

Portions of tissues easily dissociated may be teased, viz. nerves and muscles. The process of teasing is assisted by immersing the tissue for twenty-four hours in 33 per cent alcohol or some other "dissociating" fluid (*vide* p. 8). Definition is improved by the addition of acetic acid. Examination of stained sections of hardened tissues gives better results. In the case of pigmentary atrophy, lithium carmine shows up the pigment granules better than hæmatoxylin.

(C) CLOUDY SWELLING.

Cloudy swelling is best seen in cells obtained by scraping the fresh tissue or teasing in water. To distinguish cloudy swelling from fatty changes acetic acid is added. The granules due to cloudy swelling vanish, while, on the other hand, they are unaffected by fat solvents, such as absolute alcohol, ether, chloroform, etc.

(D) FATTY DEGENERATION.

Fatty degeneration can be best studied in fresh tissues, either by teasing or in sections.

The following are the micro-chemical reactions of fat particles present in the cells:—

1. They do not disappear when treated with acetic acid.
2. They resist the action of dilute caustic potash and soda (*vide* p. 11).
3. They are stained black on the addition of 1 per cent osmic acid.
4. They are dissolved by chloroform and ether.

In the last reaction the tissue should be first dehydrated in absolute alcohol, then left for some time in chloroform or ether, again washed with alcohol, and finally examined in salt solution.

Alcohol should not be used for hardening, as it gradually dissolves out the fat. When hardened in Müller's fluid, although the fat granules are preserved, they tend to run together to form larger drops. Very good results can be obtained, however, by hardening in Müller's fluid and osmic acid, as in Marchi's method (*vide* p. 135).

Flemming's solution (*vide* p. 56) gives admirable results. The tissue is hardened in this for four days, then carefully washed for twenty-four hours, then hardened in 30 per cent, 60 per cent, 96 per cent alcohol, one day in each, and lastly embedded in celloidin. If the tissues are not very carefully washed, black precipitates form. To prevent this, Flemming recommends treatment with osmic acid in the dark. The pieces are cut by a freezing microtome, and may be stained with saffranine (p. 57). It is often useful to stain a section with eosine alone for the purpose of comparison.

After staining, the sections are dehydrated in absolute alcohol, cleared in oil, and mounted in Canada balsam. The oil used for clearing must be very carefully removed from the sections. Oil of turpentine should be avoided, and the sections should not be allowed to stay long in xylol (as in paraffin embedding), because both these fluids, as well as ether and creasote, dissolve the fat which has been acted on by osmic acid. This is not dissolved by alcohol, chloroform, or oil of cloves. Solid Canada balsam, melted by warming over a flame, is used for mounting. The fat granules become somewhat diffuse when ordinary liquid Canada balsam dissolved in xylol is employed.[1]

All preparations, for which the stronger osmic acid solutions have been used for fixing and staining the fat, must be very thoroughly washed after they are taken out of the osmic acid, and before they are transferred to alcohol. If this be not done the alcohol removes the excess of osmic acid from the tissue, so that the fluid becomes more or less dark-coloured, owing to the presence of very fine particles of osmic acid. These changes in the fluid outside may also take place in the alcohol penetrating the tissue (Heidenhain). The opaque particles of osmium may then form a deposit in parts of tissues which contain no fat. Deceptive appearances of this kind may be avoided, however, by washing out the excess of osmic acid in large quantities of water before placing the tissue in alcohol.

(E) MUCOID OR MYXOMATOUS DEGENERATION.

Tissues which have undergone myxomatous changes are best examined in the fresh state. Müller's fluid should be used for hardening, as both acetic acid and alcohol cause coagulation.

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[1] Or Farrants' solution (*vide* p. 42).—ED.

Hoyer recommends fixation in corrosive sublimate and subsequent embedding in paraffin for the demonstration of mucin, though he also stains it in celloidin preparations.

Mucin stains well with all basic aniline dyes, but particularly well with methylene blue (aqueous solution).

A double stain is produced by using the variety of Thionin known as amethyst (Joh. Rud. Geigy und Co., Bâle) and Toluidine blue (two drops of a saturated aqueous solution to 5 ccm. of water). This stains the mucin bluish red to reddish violet, the rest of the tissue light blue. Mucin also stains very well by Van Giesson's method (*vide* p. 54).

(F) COLLOID DEGENERATION.

Tissues which have undergone partial colloid degeneration are hardened in alcohol or Müller's fluid. Hæmatoxylin or double-staining with hæmatoxylin and eosine may be used.

By Van Giesson's method colloid material stains a yellowish red, and sometimes bright red, so that it becomes very clearly differentiated from the rest of the tissue. This stain is not enough, however, to distinguish it from hyaline degeneration, as colloid substance often stains as bright a red as hyaline material.

(G) LARDACEOUS CHANGE.

Tissues affected by this change can be readily cut in the fresh state on account of their firmness. They may also be hardened in alcohol or Müller's fluid.

The change is recognised by the following reactions :—

a. Reaction with Iodine.

The sections are placed in a sherry-coloured iodine solution, made by diluting the ordinary Lugol's solution (*vide* p. 10) with distilled water. After three minutes they are washed in water and examined in glycerine. The parts which have undergone the lardaceous change acquire a dark brown colour, while the rest of the tissue becomes yellow.

By adding 25 per cent glycerine to the iodine solution the brown colour becomes more brilliant.

The colour is not permanent. Permanent preparations can be made, however, by the method recommended by Langhans for tissues containing glycogen (*vide* p. 68).

b. Iodine Sulphuric Acid Reaction.

If the sections which have been treated with iodine as above are now placed in 1 per cent sulphuric acid, the brown colour of lardaceous change either becomes darker or goes through a violet blue, and then a green stage. Treatment with iodine alone will sometimes produce one of these shades of colour.

The following substances give reactions with iodine like those of lardaceous change:—

1. Corpora amylacea, occurring chiefly in the prostate and central nervous system. They stain brown with iodine, but the rest of the tissue remains yellow.
2. Starch granules become blue when treated with very dilute (1 : 4-5) Lugol's solution.
3. Cellulose from plants stains yellow with iodine solution. If the specimen be now quickly irrigated with water, and then pure sulphuric acid be allowed to flow in from the edge of the cover-glass, the cellulose acquires a deep blue colour at the point where the acid is acting.[1]

c. Methyl-Aniline-Violet Reaction.

1. Stain the tissue for three to five minutes in 1 per cent methyl-aniline-violet solution.
2. Wash in distilled water, to which 1 per cent HCL. has been previously added.
3. Mount in glycerine.

The lardaceous material becomes purple-red, the rest of the tissue blue.

The stain keeps for some time in glycerine, so that sections may be mounted permanently by edging round with wax and afterwards with varnish.

The same colour reaction can be obtained by using so-called

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[1] Virchow states that under certain circumstances permanent cartilage may give a bluish colour with iodine and sulphuric acid.—*Cellular Pathology*, Eng. translation, p. 377.—Ed.

Leonhardi's ink ("Telegraphen tinte"), which contains methyl-aniline violet.

The sections must not be dehydrated in alcohol nor mounted in Canada balsam. The colour is retained, however, fairly well by mounting in a saturated solution of potassium acetate. Weigert recommends a solution of lævulose for mounting. Syrup of cane sugar is cheaper, and does quite as well.

d. Methyl-Green.

This is used in the same way as methyl-violet, but the lardaceous material stains violet, and the nuclei and the rest of the tissue green.

e. Iodine-Green.

1. Sections of fresh or hardened tissues are stained for twenty-four hours in a solution of

Iodine green	.	0·5 grm.
Distilled water	.	150·0 ccm.

2. Wash in water.

The parts affected by lardaceous change stain reddish violet, the rest of the tissues green. According to Stilling, this reaction is more reliable than that given by methyl-violet.

f. Birch-Hirschfeld's Method.

1. Stain for five minutes in 2 per cent alcoholic solution of Bismarck brown (*vide* p. 49).
2. Wash in absolute alcohol.
3. Wash in distilled water for ten minutes.
4. Stain for five to ten minutes in a 20 per cent solution of gentian violet.
5. Wash in acidified water: ten drops of acetic acid to a large watch-glass of water.
6. Mount in lævulose.

This method differentiates the lardaceous from the healthy parts of the tissues, the nuclei of which are stained brown.

g. Double-Staining with Hæmatoxylin and Eosine.

The relation of the lardaceous material to the various tissue elements should always be investigated by this method. In sections treated in this way the morbid changes may often be more readily localised than by any of the special lardaceous reactions. The lardaceous material stains red, while the nuclei of the atrophied cells, which are often not shown by the usual lardaceous stains, can easily be distinguished in the immediate vicinity of the masses of lardaceous material.

h. Van Giesson's Method.

This (*vide* p. 54) is admirably adapted for this purpose. The lardaceous material stains a rose to a reddish-brown colour.

(H) HYALINE DEGENERATION.

This change is best shown by the double stain of hæmatoxylin and eosine. In sections which have been only single stained, however, the homogeneous, glassy condition of tissues which have undergone hyaline degeneration is generally sufficiently visible.

Van Giesson's method (p. 54) is very suitable for this purpose, the hyaline material staining bright red.

(I) STAINING REACTIONS FOR GLYCOGEN.

Pieces of tissue, *e.g.* diabetic kidney, tumours, etc., should be placed in alcohol as soon as possible. Water dissolves glycogen, so that Müller's fluid cannot be used. The sections are treated with a dilute iodine solution, which stains glycogen brown red. Even in an iodine solution the effect of water is considerable, so that it is better to use (Ehrlich) a thick gum arabic solution to which 1 per cent Lugol's solution (*vide* p. 10) has been added. The sections should be mounted in this solution.

Sections may also be examined in glycerine mixed with half its volume of Lugol's solution. Another advantage of this method is that the sections are cleared at the same time.

According to Langhans, origanum oil is particularly suitable for clearing and mounting. Before mounting in origanum oil, however, the sections should not be dehydrated in pure absolute alcohol, which would remove the iodine at once, but must be treated with a mixture of four parts absolute alcohol to one part tincture of iodine (B.P.). The process is as follows:—

1. Harden as fresh as possible in absolute alcohol.
2. Stain in Lugol's solution.
3. Dehydrate in a mixture of tincture of iodine 1 part, absolute alcohol 4 parts.
4. Clear and mount in origanum oil.

(K) CALCAREOUS INFILTRATION.

Lime salts recently deposited in the tissue appear as bright granules, which dissolve when 5 per cent hydrochloric acid is allowed to flow in from the edge of the cover-glass. Parts previously opaque now become transparent, owing to the lime salts being dissolved, the calcium carbonate disappearing with the evolution of bubbles of gas, and the phosphates without. Tissues affected by early calcareous changes often stain deep blue with hæmatoxylin; this also occurs in decalcified tissues, *e.g.* the calcified cartilage-matrix in intra-cartilaginous ossification.

(L) CHANGES RESULTING IN THE FORMATION OF PIGMENT.

Hæmatoidin may be found in old hæmorrhages, either in the amorphous condition or in the form of rhombic plates. It is easily recognised by its orange-yellow or reddish colour. Besides this, amorphous yellow or dark-brown masses may be present, to which the name of "hæmosiderine" has been given, on account of its containing iron.

The presence of **Hæmosiderine**, or rather of its iron constituent, is shown by the following reactions:—

a. Potassium Ferro-cyanide Solution and Hydrochloric Acid.

The sections are placed for a few minutes in a 2 per cent aqueous solution of potassium ferro-cyanide, and then in glycerine, to which $\frac{1}{2}$ per cent of hydrochloric acid has been added. The hæmosiderine acquires a distinct blue colour.

To prepare permanent specimens which will exhibit nuclear staining at the same time, the following method should be adopted. The sections are stained in ordinary lithium carmine (*vide* p. 47) solution, to which a few drops of ferro-cyanide solution have been added. They are afterwards treated with acidified glycerine, or else washed in acid-alcohol (*vide* p. 47), transferred to water, dehydrated in absolute alcohol, cleared, and mounted in Canada balsam.

Stieda's method is well adapted for staining nuclei as well as for showing the iron reaction. It is as follows:—

1. Stain in lithium carmine for several hours.
2. Wash for a short time in distilled water.
3. Place in potassium ferro-cyanide solution for four to six hours.
4. Transfer to 1 per cent HCL. alcohol for six to twelve hours.
5. Wash in water, spirit, absolute alcohol, origanum oil, Canada balsam.

Or the iron reaction may be first obtained with the ferro-cyanide solution and acid glycerine, the sections being then washed, and afterwards stained with alum carmine.

b. Ammonium Sulphide Reaction.

1. Place the sections for five to twenty minutes in a freshly prepared solution of ammonium sulphide until they acquire a dark green or greenish-black colour.
2. Wash quickly in water.
3. Mount in glycerine, which has a feeble solvent action on ammonium sulphide.

The iron appears in the form of black or blackish-green granules.

The sections may instead be transferred from the water in which they were washed to alcohol, oil of cloves, and then Canada balsam.

Zalefski has lately proposed certain modifications of the potassium ferro-cyanide and ammonium sulphide methods. Small pieces of the tissue are treated in bulk instead of separate sections.

The method is as follows:—

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(*a*) Ammonium Sulphide.

(1) The small pieces of tissue are left in 65 per cent alcohol for twenty-four hours.
(2) They are then hardened for twenty-four hours in 96 per cent spirit, to which a few drops of strong yellow ammonium sulphide have been added. They must be shaken up from time to time.
(3) They are next hardened in absolute alcohol, to which several drops of ammonium sulphide are added.
(4) They may then be cut, etc.

The alcohol should completely fill the vessel, in order to prevent oxidation by the air. Corks must not be used, because they give themselves an iron reaction with ammonium sulphide. Pieces of tissue may be kept for a long time in alcohol containing ammonium sulphide.

(*b*) Ferro-cyanide of Potassium.

(1) Leave the small pieces in 65 per cent alcohol for twenty-four hours.
(2) Place them for two to three days in a 1 per cent solution of ferro-cyanide in 96 per cent alcohol.[1]
(3) Place them in a 1 per cent solution of ferro-cyanide in 65 per cent alcohol.
(4) Place them in 1 to 2 per cent acid-alcohol (alcohol = 96 per cent) for two to three days.
(5) Cut sections. They may afterwards be stained in carmine.

Treatment with 65 per cent alcohol (as in 3) is employed to aid the penetration of the acid used subsequently. Tissues treated in this way with potassium ferro-cyanide afterwards stain better than when treated with ammonium sulphide.

Potassium ferri-cyanide may be used in a similar way to the ferro-cyanide for ferrous compounds, and gives a blue colour. With ferric compounds, potassium sulphocyanate may also be used, and the specimens are afterwards treated with hydrochloric acid in both cases.

When examining for iron, steel needles, of course, should not be used, and care must be taken to avoid contact with materials containing iron.

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[1] Von Kahlden has been unable to obtain a solution of this strength in 96 per cent alcohol.

Quincke has stated as an objection to the use of potassium ferro-cyanide that it coagulates the albumen, and is apt to diffuse into the surrounding tissues. The latter objection is of small moment if the progress of the reaction in acid glycerine be watched under the microscope.

On the other hand, it must not be forgotten that sulphide of ammonium produces precipitates with a number of other metals similar to that with iron. Practically, silver, lead, and mercury are the only ones likely to occur.

It is, therefore, often advisable to employ both reactions, one after the other.

It must also be noted that all iron-containing pigments do not give the reactions which have been described. Sometimes the pigment is combined with the tissue in such a way that no reaction results, and in other cases the pigment may give the iron reaction to only a limited extent. This, however, does not happen when the tissues are quite fresh, or have been kept for a long time.

For merely ascertaining the situation of the pigment, the red stains, such as lithium carmine, borax carmine, or alum carmine, are the best, because the pigment stands out well against them.

Alum carmine is better than lithium carmine, because with it washing in hydrochloric acid is unnecessary.

For the preparation of hæmin crystals see Chapter XV.

[PHOSPHATES.

These may be demonstrated by Lilienfeld and Monti's method (*Zeitschrift der Phys. Chem.* Bd. xvii. 410). Sections or teased portions of the fresh tissues are soaked in a solution of ammonium molybdate. If the phosphates are abundant, the yellow colour thus produced may be visible to the naked eye. The sections are then transferred to a 20 per cent solution of pyrogallol, dissolved either in water or, preferably, in ether. They are then dehydrated, cleared, and mounted in Canada balsam. The action of the pyrogallol is to reduce the phospho-molybdate, and the resulting coloration is brown or black, according to its intensity.—ED.]

CHAPTER X

THE EXAMINATION OF PROLIFERATING AND INFLAMED TISSUES

PROLIFERATING and inflammatory tissues should be hardened in Flemming's solution, in Müller's fluid, or in alcohol. This applies also to tumours.

Müller's fluid is best for avoiding shrinkage of the tissues or for preservation of the blood *in situ.*

Alcohol should be used for hardening if bacteria are present.

For a more accurate investigation of the cells and nuclear structures contained in proliferating tissues, as, for example, tumours, they should be examined as fresh as possible in a fluid which does not affect them, such as 0·6 per cent salt solution. Von Arnold strongly recommends a dilute solution of methyl green in 0·6 per cent salt solution for the examination of cells and their nuclei. This applies to tissues which have been treated by some fixing reagent, of which Flemming's solution is the best, for showing karyokinetic figures. It is most important to examine the nuclear figures in order to decide from what kind of cells the proliferating or newly-formed tissue is developed in inflammatory formations, hyperplasias, tumours, etc.

In the case of tumours, pieces should be taken from the growing edge.

Sections should be first treated with nuclear stains. It will be found that the different kinds of cells stain in varying degrees. Leucocytes stain the darkest, and so migrated white blood corpuscles in inflammatory infiltrations can be distinguished from the cells of the original tissue, even when these resemble them in appearance.

Large granular cells (Ehrlich's **Mastzellen**) are often found in considerable numbers in chronic inflammation, especially in connective tissues. They are two or three times larger than leucocytes, and have pale nuclei. The protoplasm is coarsely granular, and the granules stain with basic aniline dyes and with the same stains which are used for bacteria, while the nucleus itself generally remains unstained.

Ehrlich's method for staining these cells is as follows:—

(1) Harden in alcohol.

(2) Stain for twelve to twenty-four hours in the following solution:—

Dahlia (in excess).	
Absolute alcohol	50 ccm.
Acetic acid	12·5 „
Distilled water	010 „

(3) Wash in water.

By **Westphal's** modification of Ehrlich's method the nuclei of cells as well as the "Mastzellen" (*vide* p. 73) in inflammatory tissues may be stained. This is as follows:—

1. Stain for twenty-four hours in the following solution:—

Dahlia, strong alcoholic solution	100·0 ccm. of each.
Alum carmine	
Glycerine	
Acetic acid	20 „

2. Wash in alcohol.
3. Oil, Canada balsam.

The nuclei of the tissue are stained red, while the granules of the "Mastzellen" and bacteria become blue.

The phenomena of **inflammation** in living tissues are generally studied in the frog's mesentery.

For this purpose a large male frog should be selected, because the sexual organs of females often make it more difficult to draw out the mesentery. Two drops of a 1 per cent aqueous solution of urari are injected under the skin covering the thigh, which should previously have been snipped with a fine pair of scissors. The frog becomes paralysed in one-half to three-quarters of an hour. An incision is made in the abdominal wall in the axillary line, the skin and muscular layer being separately divided. Lastly, the abdominal cavity is opened to the extent of about 1 cm. by a vertical incision. If bleeding occurs at any stage of the operation, it should be checked before carrying the incision deeper. Slight pressure with a piece of blotting-paper is generally sufficient.

A loop of intestine, with the mesentery attached, is now drawn out and stretched out over a ring of cork, previously cut out from a sheet of cork and fastened on a glass plate with sealing wax. The mesentery is fixed with pins, which are driven firmly through the intestine into the cork. The animal should be fully under the influence of urari, so as to prevent movement.

The frog can be placed on a sheet of glass such as is used for making plate cultivations. The cork is fastened close to one of the longer sides, so that there is room for the frog to lie on the plate by the side of the cork. The animal is then covered with moistened blotting-paper, with the exception of the part lying on the cork ring, which is to be examined under the microscope.

Generally in about a quarter of an hour's time migration of the white blood corpuscles may be seen to commence, and the process may be watched for hours if the frog is kept completely covered with wet blotting-paper. The examination may be carried on for a whole day if desired, but the injection of urari must be repeated.

The web of the foot or the tongue of the frog may be employed in the same way.

CHAPTER XI

EXAMINATION OF BACTERIA

(A) Bacteria in fluids may be examined by the following methods:—

1. Examination of Unstained Bacteria on a Slide.

A drop of the fluid is placed on a slide and covered with a cover-glass. If the fluid contains a large amount of organisms it should be diluted with distilled water.

The examination is made under a high power and with a narrow diaphragm. The addition of dilute acetic acid or 2 per cent caustic potash is useful in differentiating micrococci from proteid granules. It has no effect on bacteria, with the exception of the spirillum of relapsing fever. Similarly, bacteria are unaltered by alcohol, chloroform, and ether, while fatty particles are dissolved.

In most cases the uniform shape and size of the bacteria and their peculiar arrangement in chains, clusters, or zoogloea, are sufficiently distinguishing features.

For the examination of unstained bacteria from cultures a drop is taken from the tube with a platinum wire sterilised by heat, placed in a drop of water on a slide, and well stirred up in it. Salt solution (0·6 per cent) or broth may be used instead of distilled water. If the movements of the bacteria interfere with the examination, they may be stopped by allowing a drop of sublimate solution to flow in from the edge of the cover-glass.

2. Hanging-drop Method.[1]

This is a much more important method, as it affords a means of watching the development of bacteria while living and multiplying.

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[1] See also Delépine's method of interlamellar films. *Lancet*, 1891, p. 1303.—Ed.

A special glass slide is required with a circular, shallow depression ground in the centre. A thin layer of vaseline is smeared round the margin of the depression, so that when a cover-glass is placed on it a small air-tight chamber is formed. The cover-glass is carefully cleaned, and a small drop of the fluid to be examined is placed on the centre of it. The cover-glass is now lowered on to the slide on the layer of vaseline, so that the drop of fluid hangs down suspended from above in the hollow space. Both slide and cover-glass and any other instruments which may be used should have been carefully sterilised by heat.

A narrow diaphragm should be used in examining bacteria by this method. Great care must be taken to avoid pressing upon the cover-glass while focusing. When the examination of the drop is completed the cover-glass may be raised, and the vaseline sticking to it removed with benzole. Part of the drop is then taken off and the remainder dried, and afterwards may be stained.

To study the development of special micro-organisms from pure cultures, a drop of sterilised broth is placed on a cover-glass and inoculated from the culture with a sterilised platinum needle. In this way the examination by the hanging-drop method may be carried on for days, and in the intervals the preparation is kept in an incubator.

3. Methods for staining Bacteria.

The basic aniline colours gentian violet, methylene blue, fuchsine, and Bismarck brown (vesuvine) are the common dyes for bacteria. Aqueous solutions are generally employed. As aqueous solutions do not keep well, it is better to prepare beforehand strong alcoholic solutions of these dyes, which can be added to distilled water when required, so as to form a fresh aqueous solution containing from 1 to $1\frac{1}{2}$ per cent of the stain. The staining power of aniline colours may be increased as follows :—

(*a*) By warming either for a long time in an incubator, or for a short time over a spirit-lamp, until vapour rises from the upper surface of the fluid.

(*b*) By the addition of caustic potash. Koch, in his original method for staining tubercle bacilli, employed this in addition to heating.

At present caustic potash is used chiefly in Löffler's methylene blue solution, which is widely employed for staining micro-organisms, because of its wide range of application and its permanency.

(*c*) By dissolving the stain in aniline water. This is prepared by adding five parts of aniline oil to 100 parts of water and thoroughly shaking. The milky fluid produced in this way is then filtered. It should come through quite clear and transparent. The stain may be dissolved in this solution directly, or may be added as a concentrated alcoholic solution until distinct opalescence results.

Aniline-water solutions of fuchsine and of gentian violet are those most commonly used.

(*d*) The addition of five per cent carbolic acid solution increases the action of aniline dyes in the same way as aniline water. Carbolic acid solutions of aniline dyes stain remarkably well, and can be kept for any length of time.

The excess of the stain can generally be got rid of by simple washing in a large quantity of distilled water.

The following reagents may also be used for removing the excess of the stain:—

(1) Alcohol.
(2) Dilute acetic acid ($\frac{1}{2}$ to 1 per cent).

Both these decolourise sections more thoroughly than water, and define the nuclei more sharply. For the latter purpose the acetic acid is often used before the alcohol.

(3) Iodine dissolved in potassium iodide solution, one part iodine, two parts pot. iodide, 100 parts water.
(4) Mineral acids 3 to 10 per cent.

Both these solutions rapidly decolourise some species of bacteria, while other kinds of bacteria, being more retentive of the dye, become better differentiated.

(5) Various salts for decolourising nuclei, sodium and potassium carbonates, liquor ferri sesquichloridi, potassium bichromate and permanganate, palladium chloride, lithium carbonate, etc.

(6) Acid aniline dyes, tropæolin and fluorescin, if added to the alcohol employed for decolourising, produce a greater effect than can be obtained by alcohol alone.

For most purposes the various basic aniline colours mentioned above stain equally well.

The following, as already stated, will be found very generally useful.

Löffler's Solution of Methylene Blue.

Concentrated alcoholic solution of methylene blue	30 ccm.
Caustic potash, 0·01 per cent solution .	100 „

This, like all methylene blue solutions, keeps better than other aniline dyes.[1]

Preparing and Staining Cover-glass Preparations.

To stain bacteria in fluids, a thin film should be smeared on a cover-glass by means of a platinum loop, or a drop of the fluid is placed on one cover-glass; a second cover-glass is pressed on this, and they are then drawn apart. The cover-glass, with the smeared surface uppermost, is allowed to dry in the air, and is then slowly passed three times through the flame of a spirit-lamp. Care must be taken that the preparation has become quite dry in the air before it is fixed by heating.

The cover-glass, held between the thumb and forefinger, is placed on the staining fluid, so that it floats on it with the dried film on its under surface. Another method is to place a drop or two of the stain on the cover-glass itself with a glass rod or pipette. After two or three minutes it is washed by moving it to and fro in a dish containing water, until no more stain comes out, or by playing on the stained side with a jet of water from a wash-bottle. The cover-glass is then very carefully dried with blotting-paper, and lastly, held over the flame of a spirit-lamp for a short time, to ensure complete dryness. The Canada balsam will become turbid if any trace of water remain.

The cover-glass is then fixed to the slide with a drop of Canada balsam. Preparations which are not to be kept permanently may be mounted in a drop of water placed on the slide. Specimens mounted in Canada balsam are more successful.

To stain bacteria without staining nuclei, etc., the cover-glass is taken out of the staining fluid, and placed for one minute in a saturated solution of potassium carbonate, diluted with an equal quantity of water. Then wash in water, etc.

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[1] *Vide* note, p. 61.

Summary of the various stages in staining cover-glass preparations:—

1. Spread out the fluid on the cover-glass and allow it to dry.
2. Draw it three times through a flame.
3. Stain for one to three minutes.
4. Wash in water.
5. Dry between blotting-paper.
6. Canada balsam.

Aqueous solutions of fuchsine, gentian violet, methylene blue, and Bismarck brown may be employed for staining in this manner. Gentian violet gives good results, especially where reagents, such as carbolic acid, aniline water, etc., are employed to intensify its action.

Löffler's methylene blue solution is very useful, as are also the various staining solutions containing carbolic acid. In many cases Gram's method is more suitable.

Gram's Method.

1. Stain for two to five minutes in a saturated solution of gentian violet in aniline water.
2. Place the specimen for one to one and a half minutes in iodine solution (iodine 1 part, potassium iodide 2 parts, water 300 parts). It becomes quite black in this.
3. Decolourise in alcohol until the original black colour vanishes and the specimen turns quite pale gray.
4. Mount in Canada balsam.

Günther showed that alcohol decolourises more rapidly and completely when 3 per cent of nitric acid is added to it; and another advantage of this is that precipitates of colouring matter are less likely to occur. After the iodine solution, the preparations are placed in pure absolute alcohol, then for ten seconds in nitric acid alcohol, and then again in pure alcohol. According to **Ribbert**, decolourisation is very rapidly produced by the addition of ten to twenty parts of acetic acid to the alcohol employed.

Gram's method has two important advantages:

Firstly, the whole of the cell constituents of the tissue are decolourised except the karyokinetic figures, the granules in the large granular cells known as "Mastzellen," the cells of the corneous layer, the epidermis, and the serous coat of organs. Bacteria are stained a deep blue colour, and therefore are much more sharply defined. Bismarck brown may be used as a contrast stain.

Secondly, certain kinds of bacteria retain the dye, and are thus differentiated from others resembling them morphologically which are decolourised.

The following micro-organisms stain by Gram's method—**Bacilli**—Tubercle, leprosy, tetanus, mouse septicæmia, anthrax, and swine erysipelas. **Cocci**—Pneumonococcus of Fränckel-Weichselbaum, streptococcus pyogenes and erysipelatosus, staphylococcus pyogenes aureus, albus, citreus and flavus, and micrococcus tetragenus.

The following become decolourised—**Bacilli**—typhoid fever, diphtheria, glanders, malignant œdema, rauschbrand (charbon symptomatique); Friedländer's encapsuled pneumonia bacillus; Koch's comma cholera bacillus; bacillus of chicken cholera, rabbit's septicæmia, hog cholera, cattle plague, and wildseuche; **Gonococci**; **spirillum** of relapsing fever.

Staining the Flagella and Cilia in Bacteria.

In **Löffler's method** a mordant is employed, which is prepared as follows:—

(1) An aqueous solution of ferrous sulphate, saturated in the cold, is added drop by drop to 10 ccm. of an aqueous solution of tannin (20 : 80) until the liquid acquires a deep violet colour; 1 ccm. of an aqueous or alcoholic fuchsine solution is then added (methyl violet or logwood solution = 1 to 8 do not act so well). The dried film on the cover-glass is treated with this, and heated over a flame until vapour arises from it, and is left for one or one and a half minutes longer in contact with the fluid.

If the fluid under examination contains mucin, albumen, or gelatine, they must be got rid of as completely as possible by repeated washing in water.

The cover-glass preparation should be passed through the flame in the ordinary way before using the mordant; its action is prevented, however, by overheating.

In the case of acid-producing bacteria it is advisable to add an alkali, such as a few drops of a 1 per cent NaOH solution, while with those which give rise to alkaline bodies it is better to add acid (H_2SO_4). The amount of acid or alkali to be added must be determined by experience.

(2) Wash in water, afterwards in absolute alcohol.

(3) Stain with a saturated aniline-water fuchsine solution, which has been neutralised by the addition, drop by drop, of a 1 per cent NaOH solution. The NaOH solution should be added until a layer of the previously transparent fluid, several centimetres thick, begins to become opaque. A drop of this staining solution is then placed on the cover-glass, and the whole warmed for about a minute until vapour arises.

[(*a*) Mr. E. C. **Bousfield** has kindly furnished me with the following additional directions for the staining of flagella by Löffler's method:—

The reaction of the ferrous sulphate solution is of importance; it should not be acid, and is best made from old partially oxidised ferrous sulphate. The mordant must be freshly made. Mr. Bousfield uses 10 cc. of the tannic acid solution, to which, after warming, 5 cc. of the ferrous sulphate solution are added, drop by drop, and without any addition of fuchsine or methyl-violet.

In making the cover-glass preparations use very recent cultivations, preferably those of eight or ten hours' growth only, as after twenty-four hours the flagella are shed. An emulsion of the growth should be made in ordinary tap water, and a little of this added to a drop of distilled water on a cover-glass perfectly grease-free, and then spread out by a single movement of the platinum wire into a delicate film. It is better to dry the film in air than to hold it over the flame; it may then be fixed over the flame, held between the finger and thumb, so as to avoid overheating. The freshly prepared mordant is then used as described in the text, and the cover-glass rinsed thoroughly with distilled water, and then with spirit. Lastly, after staining with fuchsine or methyl-violet it is best to wash with distilled water only, and not subsequently with spirit.—Ed.]

[(*b*) **Nicolle and Morax's** modification of Löffler's method.

Place a drop from the culture in a watch-glass of tap water. A drop of the diluted fluid is then placed on a cover-glass and allowed to dry in the air (protected from dust). A drop of Löffler's mordant is placed on the cover-glass preparation, and heated for ten seconds until vapour arises, and it is then washed in water. The application of the mordant, the warming, and washing are repeated three times.

A drop of Ziehl's fuchsine solution (*vide* p. 100) is then placed on the cover-glass and heated once or twice for a quarter of a minute. Wash in water and examine in water. If the staining is successful, dry and mount in Canada balsam.—Ed.]

(B) **Staining Bacteria in Sections.**

Some staining method is always employed when bacteria are examined in sections of tissues.

For this purpose the sections are transferred directly from absolute alcohol to the staining solution. Gentian violet is very suitable for most cases.

As a rule, the same methods may be employed for sections as for cover-glass preparations, though certain modifications are necessary.

(1) Sections require longer staining than cover-glass films.

(2) Heat should be applied while staining, either in an incubator or over the flame of a spirit-lamp. The staining fluid is warmed over a flame until vapour begins to come off from the surface.

(3) When the nuclei have lost their colour, or become indistinct, owing to the decolourising process, they can be defined again by means of a contrast stain, such as lithium carmine, picro-carmine, or Bismarck brown.

(4) To prevent excessive decolourisation during dehydration in alcohol, a small quantity of the same stain may be added to the alcohol employed. The alcohol then removes the water without taking out more of the colour. As a rule, however, this precaution is unnecessary. If the sections have been well stained a slight loss of colour is rather an advantage than otherwise.

(5) Clove oil should not be used for clearing the sections, as it dissolves out aniline dyes, and to a certain extent spoils the colour. Origanum oil is better, but xylol will be found best in most cases.

The following are some of the methods in use for staining bacteria in sections:—

Löffler's Method.

1. Stain for three to five minutes in Löffler's methylene blue (*vide* p. 79).
2. Ten to thirty seconds in 0·5 to 1 per cent acetic acid.
3. Dehydrate in alcohol, xylol, Canada balsam.

A stronger decolourising solution is prepared by adding tropæolin to the acetic acid until it acquires a light yellow colour.

Staining with Gentian Violet.

1. Stain in a 2 per cent aqueous or alcoholic solution of gentian violet until the sections acquire a dark violet colour.
2. Wash in absolute alcohol until they become light violet.
3. Xylol, Canada balsam.

This method gives excellent results, especially with sections containing anthrax. The nuclei stain a distinct light violet colour, against which the bacteria are sharply defined, owing to their darker tint.

It will sometimes be found of advantage to place the sections after staining them, and before washing in alcohol, in 5 per cent acetic acid for ten to twenty seconds.

Pfeiffer's Fuchsine Method.

(1) Harden in alcohol.
(2) Stain for half an hour or longer in carbol-fuchsine solution (p. 78), one part to twenty of distilled water.
(3) Wash in a dish of distilled water, to which one drop of acetic acid has been added, until the section acquires a grayish violet colour.
(4) Alcohol, oil, Canada balsam.

Gram's Method (*vide* p. 80).

Sections must be left longer in the staining fluid than cover-glass films. After staining, the sections may either be placed directly in the iodine solution, or after remaining for a very short time in 0·6 per cent salt solution. They should be removed on a lifter, as they are otherwise very apt to curl up.

A contrast stain had better be employed, for which purpose Bismarck brown may be used in the following way:—

1. After decolourising in alcohol, stain for two to five minutes in an aqueous or alcoholic solution of Bismarck brown (*vide* p. 49).
2. Wash in absolute alcohol until the sections become a yellowish-brown colour.
3. Xylol, Canada balsam.

A nuclear stain, such as picro-carmine (*vide* p. 48) or lithium carmine (p. 47), may be employed before staining for bacteria. This method is better, on the whole, because when Bismarck brown is used as a subsequent stain many bacteria become indistinct.

Weigert's Method.

1. Harden the tissues in absolute alcohol.
2. Stain for five to fifteen minutes in a saturated solution of gentian violet in aniline water.
3. Wash for a short time in 0·6 per cent salt solution.
4. Remove the section from the salt solution on a lifter or a glass slide, and dry it carefully with blotting-paper.
5. Dip the lifter or slide bearing the section in iodine solution for one to two minutes.
6. Dry with blotting-paper.
7. Decolourise in aniline oil until no more colour is taken up.
8. Remove the aniline oil with xylol.
9. Canada balsam.

Excellent results are obtained by this method. Bacteria alone (and fibrin filaments, if present) are stained deep blue.

The tissue elements of the sections can be shown up by previously staining for three minutes in lithium carmine (*vide* p. 47). They are then transferred from the water used to remove the acid-alcohol to an aniline-water solution of gentian violet. They are stained in lithium carmine first, because in Weigert's method they must not be dehydrated in alcohol.

Kühne's Methylene Blue Method.

1. Transfer the sections from alcohol to this solution :—

Methylene blue . . .	1·5 ccm.
Absolute alcohol . . .	10·0 „
5 per cent solution of carbolic acid .	100·0 „

Leave them in this for a half to two hours—the latter in the case of leprosy bacilli (*vide* p. 106).

2. Wash rapidly in water.
3. Wash in acidulated water, 2 drops hydrochloric acid to 100 of water, until the sections become a pale blue colour.
4. Wash in a solution of lithium carbonate; six to eight drops of a concentrated solution of lithium carbonate to 10 ccm. water.
5. Transfer to pure water for three to five minutes.
6. Leave in absolute alcohol for a short time.
7. Place them in methylene blue aniline oil. (As much methylene blue as will lie on the point of a knife-blade is dissolved in 10 ccm. aniline oil; a small quantity of this solution is added to pure aniline oil until the required depth of colour is obtained).
8. Pure aniline oil.
9. Transfer to an ethereal oil, such as terebene.
10. Xylol.
11. Canada balsam.

(*N.B.*—Terebene is obtained by repeated distillation of a mixture of oil of turpentine and sulphuric acid.)

This somewhat complicated method of Kühne's is applicable to many varieties of bacteria and bacilli which otherwise stain with difficulty. After the bacteria are stained, a sharp definition of the nuclei may be obtained by washing the sections in a very dilute aqueous solution of chlorhydrin blue in place of acidulated water.

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Unna's Dry Method.

Many bacteria are completely or partially decolourised by dehydrating the sections in alcohol. This is obviated by Unna's method.

The section is transferred from the decolourising fluid into water, and is there spread out on a glass slide. Pieces of blotting-paper are then pressed upon it, so as to remove as much moisture as possible, and the slide is warmed rapidly over a flame until the section is completely dried. When cool it is mounted in Canada balsam.

Preparation of Sections of Gelatine Cultures.

Several methods have been described recently for preparing sections of gelatine "stab" cultures. The gelatine or agar culture is removed from the tube either by cutting it out, or by gently warming, or by breaking the tube.

The medium surrounding the stab is hardened in 85 to 95 per cent alcohol, and afterwards fixed on a cork and cut with the microtome.

Neisser recommends that the culture should be first placed in a 1 per cent potassium bichromate solution for from one to eight days, according to size, and at the same time exposed to the action of light, then thoroughly washed in water, and hardened first in 70 per cent and then in 95 per cent alcohol.

The ordinary solutions of the aniline dyes are used for staining. Gelatine and agar themselves decolourise in alcohol quite as well as in aniline oil. Plate cultures may be treated in the same way as test-tube cultures.

Methods for staining Spores.

Spores are seen as clear, bright, highly-refractive granules, situated either in the middle or at one end of the bacillus. They stain much less readily than fully-developed micro-organisms, and with ordinary bacterial stains appear as colourless clear bodies. When once stained, however, they are less easily decolourised than bacteria. The various methods of staining spores depend on this property.

They should be stained for a long time with the aid of heat in aniline-water fuchsine or in carbol-fuchsine (*vide* p. 78), then rapidly decolourised with a mineral acid. The bacteria lose their colour, while the spores retain it.

After washing in water, a contrast stain such as methyl blue should be used to stain the decolourised bacteria. The spores retaining the fuchsine appear red, and are thus differentiated from the bacteria, which are stained blue. At present spores can only be stained with certainty in cover-glass preparations.

The process may be divided into the following stages:—

1. Prepare a cover-glass film of the fluid to be examined, dry it in the air, and then draw it three times through a flame.
2. Stain for twenty to forty minutes in warm aniline-water fuchsine or carbol-fuchsine.
3. Decolourise for one minute in 5 per cent hydrochloric acid.
4. Wash in water.
5. Stain for two minutes in Löffler's methylene blue (*vide* p. 79).
6. Wash in water.
7. Dry between blotting-paper.
8. Canada balsam.

Möller's Method for staining Spores.

1. The cover-glass film, dried in the air, is fixed by passing it three times through a flame, or by immersing it in absolute alcohol for two minutes.
2. Immerse in chloroform for two minutes.
3. Wash with water.
4. Place it for a half to two minutes in a 5 per cent chromic acid solution.
5. Wash thoroughly in water.
6. Place a drop of carbol-fuchsine on the cover-glass, and heat for one minute over a flame, allowing it to bubble once.
7. Wash off the carbol-fuchsine, decolourise in 5 per cent sulphuric acid.
8. Thoroughly wash with water.
9. Stain for thirty seconds in an aqueous solution of methylene blue or malachite green.
10. Wash, dry; Canada balsam.

Löffler's method for showing flagella in bacteria (*vide* p. 81) may also be used for staining spores.

The spores alone may be stained by drawing the cover-glass ten to thirty times through a flame, or by heating it to 200° C. in a steriliser, and then treating with an aqueous staining solution—preferably methylene blue. The nuclei and bacteria lose the power of staining, owing to the action of heat, while the spores stain readily.

SUMMARY OF METHODS FOR STAINING DIFFERENT PATHOGENIC MICRO-ORGANISMS

Micrococci of Pus.

The various kinds of micrococci commonly present in pus, such as staphylococcus pyogenes aureus, citreus, flavus, and albus, and streptococcus pyogenes, stain readily with ordinary aqueous solutions of aniline dyes, especially gentian violet and Löffler's methylene blue.

By Gram's method (*vide* p. 80) they all stain equally. It has the advantage of decolourising the pus corpuscles, and so prevents any interference with the sharp definition of the microbes. This method may also be employed for the purpose of differential diagnosis between cocci of similar appearance.

Sections may be satisfactorily stained by Gram's method, preceded by staining with lithium carmine, and followed by Bismarck brown or Weigert's stain (*vide* p. 85).

These micro-organisms occur not only in abscesses and diffuse suppurations, but also in osteomyelitis, pyæmia, infective endocarditis, perimetritis, purulent meningitis, etc.

Micrococcus of Erysipelas.

The streptococcus of erysipelas, which closely resembles streptococcus pyogenes morphologically, cannot be distinguished from it by its reactions to stains.

It stains readily by Gram's method.

Gonococcus.

The gonococcus is found for the most part inside pus cells, and this characteristic situation considerably aids the diagnosis.

Cover-glass preparations should be made from pus taken from mucous surfaces, such as the urethra, cervix uteri, the Fallopian tubes, or the conjunctiva.

Gonococci can be detected after a considerable time in stains on linen from gonorrhœal discharges. The scaly surface of the stain should be scraped off, soaked in water, and then stained on a cover-glass.

Aqueous solutions are used for staining. Gonococci are decolourised by Gram's method, a fact of importance for differential diagnosis.

The following is a method for double-staining :—

1. Stain for two to three minutes in a warm alcoholic solution of eosine.
2. Remove the excess of eosine with blotting-paper.
3. Stain for half a minute in an alcoholic solution of methylene blue.
4. Wash in water.
5. Dry with blotting-paper ; Canada balsam.

The gonococci are stained blue, the cells red.

J. Schütz recommends the following methods :—

1. Stain the dried cover-glass preparation for five to ten minutes in a saturated solution of methylene blue in 5 per cent carbolic acid.
2. Three seconds in dilute acetic acid (acid. acetic. dil. five drops, water twenty).
3. Wash thoroughly in water.
4. Stain in a very dilute solution of saffranine.

For sections **Touton** recommends staining with carbol-fuchsine, followed by washing in alcohol.

Pneumonococcus (Fraenkel-Weichselbaum).

The lancet-shaped diplococcus of Fraenkel-Weichselbaum is found most frequently in pneumonia. It is surrounded by a capsule which does not stain by ordinary methods. It occurs not only in the lungs in croupous pneumonia, but can be also frequently detected in the vegetations of recent endocarditis and the pus in cerebro-spinal meningitis. It stains with the ordinary aqueous solutions. Carbol-fuchsine (fuchsine 1, 5 per cent carbolic acid

100) is particularly useful. After staining for several hours in this solution the coccus becomes a deep red, and the capsule a light reddish tint.

To demonstrate the capsule refer below to Friedländer's encapsuled bacillus.

Fraenkel-Weichselbaum's pneumonococcus stains by Gram's method, and is thus distinguished from Friedländer's encapsuled bacillus.

Friedländer's Encapsuled Bacillus.

Friedländer's bacillus also stains readily in aqueous solutions, the capsule remaining unstained. It is decolourised by Gram's method.

The capsule may be stained in sections in the following way:—

1. Stain for twenty-four hours in the following solution, which should be kept warm:—

Strong alcoholic solution of gentian violet	50·0	ccm.
Acetic acid	10·0	,,
Distilled water . . .	100·0	,,

2. Wash in 1 per cent acetic acid.
3. Alcohol.
4. Canada balsam.

The capsule stains a pale blue colour, the central part becoming dark blue. Decolourisation of the capsule differentiates it from the bacillus, which retains its colour.

Friedländer recommends the following method for staining the capsules in cover-glass preparations:—

1. Draw the cover-glass three times through the flame, and then place it in acetic acid for a few minutes.
2. Remove the acetic acid by blowing on the cover-glass through a glass tube, and quickly drying it in the air.
3. Stain for some seconds in a saturated solution of gentian violet in aniline water.
4. Wash in water, dry between blotting-paper; Canada balsam.

Ribbert's method for staining the capsule :—

1. Stain for a few minutes in a warm saturated solution made as follows :—

Dahlia.

Alcohol . . .	50·0 ccm.
Acetic acid . . .	12·5 „
Water . . .	100·0 „

2. Wash in water, dry ; Canada balsam.

The bacteria stain dark blue, the capsules light blue. If overstained, the capsules also become dark blue, and cannot be distinguished from the bacilli.

Sarcinae Ventriculi.

These stain with aqueous solutions of the aniline dyes. As they readily overstain, it is best to employ dilute solutions of Bismarck brown.

Anthrax Bacillus.

This stains well with the ordinary aqueous solutions of aniline dyes and by Gram's method.

Sections should be stained first in lithium carmine, and then by Gram's method, followed by either Bismarck brown (*vide* p. 49) or Weigert's stain (*vide* p. 85).

Single staining in a strong aqueous solution of gentian violet followed by washing in absolute alcohol (*vide* p. 83) gives very good results. The nuclei stain a light blue colour, the bacilli a dark blue. The methods already given (p. 87) may be employed for staining the spores.

Bacillus of Malignant Œdema.

It very much resembles anthrax bacillus in general form, but is thinner. It stains with aqueous solution of gentian violet, and is decolourised by Gram's method.

Bacillus of Diphtheria.

A number of different forms of cocci and bacilli staining with ordinary dyes can be found in diphtheritic membranes. Löffler has described, besides, a bacillus staining particularly well with his methylene blue solution, and with the usual aqueous solutions, and which bears an ætiological relation to diphtheria. Löffler's bacillus is decolourised by Gram's method.

Bacillus of Rhino-scleroma.

The bacillus found in Rhino-scleroma possesses an envelope like that of Friedländer's encapsuled bacillus, from which it can be clearly distinguished by the fact that it stains by Gram's method.

Wolkowitsch's methods for staining the bacillus:—

A. In Sections:—

(1) Stain in aniline-water gentian violet for twenty-four to forty-eight hours.
(2) Wash in water.
(3) Place in iodine solution for one to four minutes.
(4) Transfer to absolute alcohol.
(5) Oil of cloves, which will also decolourise the sections to a certain extent.

A solution of picric acid in water and alcohol may be used instead of the iodine solution. The sections should be left in this for a few seconds only, as it decolourises much more rapidly.

The capsules stain well only in tissues hardened in alcohol.

The hyaline masses occurring in Rhino-scleroma stain deeply by Wolkowitsch's method, with methyl-violet, gentian violet, methylene blue, and fuchsine, less deeply with saffranine, and do not stain at all with hæmatoxylin, although they are readily stained with eosine. Double-staining with hæmatoxylin and eosine will be found very useful for this reason.

B. In Cover-glass Preparations from Cultures.

(1) Dry the film, etc.
(2) Several seconds in 1 per cent acetic acid.
(3) Dry.
(4) One minute in a strong aniline-water solution of gentian violet.
(5) Wash in water.

(6) A few seconds in 1 to 2 per cent solution of eosine.
(7) 60 per cent alcohol.
(8) Water.
(9) Dry; Canada balsam.

Stepanow recommends the following method for sections:—

(1) Stain for a quarter to one hour in carbol-gentian violet, or for twenty-four to thirty-six hours in Löffler's methylene blue.
(2) Treat for a very short time with alcohol containing $\frac{1}{4}$ to $\frac{1}{5}$ per cent acetic acid, or with alcohol alone.

Bacillus of Tetanus.

This stains readily in aqueous solutions of aniline dyes, and also by Gram's method.

Rauschbrand Bacillus (Charbon symptomatique).

This stains readily in the ordinary aqueous solutions, but is decolourised by Gram's method.

Glanders Bacillus (Malleus).

This does not stain by Gram's method.

Cover-glass preparations can be stained by simple aqueous solutions, but even these only feebly. Alkaline solutions stain much better, as does also an aniline-water solution of gentian violet to which an equal volume of caustic potash solution (1 : 10,000) has been added. This solution should be freshly prepared each time. The preparation can be quickly decolourised by placing it, before washing in water, in dilute (1 per cent) acetic acid, to which tropæolin (*vide* p. 79) has been added. The advantage of this is that the cell protoplasm becomes quite decolourised, the nuclei rather less so, and a better definition of the bacilli is thus obtained.

Löffler's method is as follows:—

(1) Stain for five minutes in an alkaline solution of methylene

blue or in an aniline-water solution of gentian violet, to which caustic potash has been added.

(2) One second in 1 per cent acetic acid, coloured light yellow with tropæolin.
(3) Wash quickly in distilled water.
(4) Alcohol, oil, Canada balsam.

Sections may be stained in the same way, but instead of using tropæolin and acetic acid as decolourising agents, it is better to leave them for five seconds in the following solution :—

Strong sulphurous acid .	2 drops.
5 per cent oxalic acid .	1 drop.
Distilled water . .	10 ccm.

It is advisable to place the sections immediately after staining for some minutes in caustic potash solution, 1 : 10,000.

It is not possible by this method to stain the glanders bacilli quite separately from the rest of the tissue.

Kühne's methylene blue method (*vide* p. 86) may also be used for staining the glanders bacillus.

This may be employed as a double stain by adding saffranine to the oil of turpentine used for clearing the sections.

Schütz's method for staining the bacilli in sections is as follows :—

(1) Stain for twenty-four hours in this solution,

Strong alcoholic solution of methylene blue } equal
Caustic potash solution 1 in 10,000 } parts.

(2) Wash in acidified water.
(3) Five minutes in 50 per cent alcohol.
(4) Five minutes in absolute alcohol.
(5) Cedar oil, Canada balsam.

All these methods are open to the objection that the tissue itself frequently becomes too deeply stained, or in decolourising the tissue sufficiently the bacteria may also partly lose their colour.

With a view to obviating this, **Noniewicz** recommends the following method :—

(1) Stain for two to five minutes in Löffler's methylene blue.
(2) Wash in distilled water.

(3) Decolourise for one to five seconds, according to the thickness of the sections, in a mixture of

0·5 per cent acetic acid	. . .	75 parts.
0·5 per cent aqueous solution of tropæolin (00)		25 „

(4) Wash in distilled water.
(5) Spread the section on a slide and dry it, first with blotting-paper, and then in the air or over a spirit-lamp.
(6) Clear by dropping xylol upon it (oil of cloves, origanum and aniline oil must be avoided).
(7) Canada balsam.

The glanders bacilli stain dark blue to nearly a black colour; the tissue is stained light blue.

Nikiforoff's method (*vide* p. 102) for staining the spirilla of recurrent fever may also be employed for staining sections containing glanders bacilli.

Bacillus of Mouse Septicæmia.

Bacillus of Swine Erysipelas.

These closely resemble one another in their morphological features and behaviour towards staining solutions. They stain readily with aqueous solutions and by Gram's method.

Bacillus of Typhoid Fever.

This may be found in the intestinal mucous membrane, Peyer's patches, the mesenteric glands, the spleen, liver, and in the fæces. It is not stained by Gram's method.

The bacillus can be readily stained in cover-glass preparations with the ordinary aqueous solutions, especially fuchsine.

Warmth accelerates the staining. Wash with water, not alcohol.

This bacillus closely resembles in morphological features a number of bacilli of putrefaction, but can be distinguished from them in sections by the fact that it becomes more sharply stained. For staining sections, Löffler's methylene blue or carbol-fuchsine is the best. The sections should be left for twenty-four hours in the staining fluid, and simply washed in water, as the bacilli are readily decolourised by alcohol.

Bacillus of Syphilis.

Lustgarten's Bacillus—Method of staining:—

(1) Stain in aniline-water gentian violet for twenty-four hours at the ordinary temperature, and then for two hours in an incubator at 40° C.
(2) Wash for three to five minutes in absolute alcohol.
(3) Decolourise:—

  (*a*) For ten seconds in a $1\frac{1}{2}$ per cent aqueous solution of potassium permanganate, afterwards—
  (*b*) For a few seconds in an aqueous solution of pure sulphurous acid.

(4) Wash in water.
(5) Alcohol, oil of cloves, Canada balsam.

It will often be found, after washing the section in water, that it is not completely decolourised. The decolourising process (3, *a* and *b*, 4) must be repeated again in this case.

The sections may afterwards be stained with saffranine.

A shorter method is that of **Giacomi**:—

(1) Stain for several minutes in hot aniline-water fuchsine.
(2) Wash in a very dilute aqueous solution of ferric chloride (a few drops of the latter to a large watch-glass of water).
(3) Decolourise in a strong solution of ferric chloride.
(4) Wash in absolute alcohol.

Doutrelepont stains for forty-eight hours in an aqueous solution of methyl-violet, and decolourises in the same way as Giacomi.

The bacilli present in the smegma præputii also stain by Lustgarten's method.

Tubercle Bacillus.

The various methods of staining tubercle bacilli resemble in some ways those already described (p. 87) for staining spores. The bacilli, when they are once stained with a particular dye, retain their colour when treated with mineral acids, while all the other constituents of the tissue, as well as other forms of bacteria, become decolourised. If the section is afterwards stained with an aqueous solution of some aniline dye which is readily distinguished from the first, a contrast stain is obtained. This stains the decolourised nuclei, bacteria, etc., and leaves the tubercle bacilli unaffected.

The staining fluid originally introduced by Koch contained caustic potash. Carbolic acid or aniline oil is now substituted for this. Aniline-water fuchsine, or carbol-fuchsine with methyl-blue as a contrast-stain, is generally used, or aniline-water gentian violet, or carbol-gentian violet with contrast-staining in Bismarck brown.

Ehrlich suggested that the tubercle bacillus possesses a cavity into which alkaline but not acid solutions can penetrate. This supposition, although it cannot be proved, affords the best explanation of the fact that tubercle bacilli, when once stained with alkaline solutions, resist the action of acids.

Warmth greatly accelerates the staining of tubercle bacilli.

Quite recently, staining for tubercle bacilli has been considerably simplified by mixing a mineral acid with the methylene-blue solution, so that decolourisation and contrast-staining are produced in one operation (B. Fraenkel, Gabbet).

It has also been shown recently that carbol-fuchsine even when not warmed stains tubercle bacilli sufficiently deeply in a short time.

Sections are treated in the same way as cover-glass preparations, but should be left for twenty-four hours in the stain, and towards the end of the time in an incubator.

To examine tubercle bacilli in sputum, the more caseous portions should be taken with a pair of forceps and divided upon a cover-glass with this, or by pressing a second cover-glass upon it. It is then dried in the air, drawn three times through a flame, and stained by one of the methods given below.

Scattered tubercle bacilli contained in sputum or other liquid media[1] may be collected for examination by one of the two following methods.

A. Biedert's Sediment Method.

Two tablespoonfuls of water are added to 15 ccm. of the sputum to be examined, and this is then stirred up with four to eight drops (not more) of caustic soda according to the viscosity of the sputum. It is next heated in a dish, keeping it stirred all the time, and four to six tablespoonfuls of water are gradually added until a thin liquid is produced. It is allowed to stand for two days in a conical vessel, the fluid is poured off from the sediment, which can then be examined.

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[1] For detecting tubercle bacilli in urine *vide* footnote, p. 132.

As the sediment loses much of the adhesive power of the original sputum, it is as well to preserve some of the sputum with which to fasten it on to the cover-glass.

B. Stroschein's Sediment Method.

5 to 10 ccm. of the sputum are mixed, according to its consistency, with one to three times its volume of a mixture of boracic acid solution and water (one part of the former to three parts of water), and is then shaken vigorously for one minute. The fluid settles in twenty-four to forty-eight hours, and the sediment can then be examined after pouring off the supernatant liquid.

To detect tubercle bacilli in tissues for purely diagnostic purposes where the relations of the bacilli to the various constituents of the tissues are of no importance, cover-glass films should be prepared from scrapings of the tissue.

This is much simpler and quicker than preparing sections. If the examination of the scrapings proves unsuccessful, sections should then be prepared. The following are some of the best methods for staining tubercle bacilli :—

I. **Ehrlich's Method** (with the addition of heat).

1. Stain for three to five minutes in aniline-water fuchsine or aniline-water gentian violet (*vide* p. 98).
2. Decolourise in 20 per cent nitric acid for a half to one minute.
3. Wash in 70 per cent alcohol until no more colour comes out.
4. Stain in methylene blue (or Bismarck brown if gentian violet is used) for one and a half to two minutes.
5. Wash in water.
6. Dry with blotting-paper.
7. Canada balsam.

Note.—Sections should remain longer in the stain than cover-glass preparations, and after washing in water should be treated with alcohol, and then xylol, and finally mounted in Canada balsam.

II. Ziehl-Nielsen Method.

1. Stain for three to five minutes in a warmed solution made as follows :—

Fuchsine	1·0 grm.
Alcohol	10·0 ccm.
Strong carbolic acid	5·0 „
Distilled water	100·0 „

2. Decolourise in 5 per cent sulphuric acid, or 15 per cent nitric acid.
3. Wash in 70 per cent alcohol.
4. Stain for one and a half to two minutes in an aqueous solution of methylene blue.
5. Wash in water.
6. Dry with blotting-paper.
7. Canada balsam.

Note.—Treat sections with alcohol, xylol, Canada balsam.

III. Gabbet's Method.

1. Stain for two minutes, without warming, in the following solution :—

Fuchsine	1·0 grm.
Alcohol	10·0 ccm.
5 per cent carbolic acid solution	100·0 „

2. Wash in water.
3. Leave for one minute in the following solution, which decolourises and at the same time acts as a contrast stain :—

Methylene blue	2·0 grms.
25 per cent sulphuric acid	100·0 ccm.

4. Wash in water.
5. Dry, Canada balsam.

Sections are treated with alcohol, xylol, and Canada balsam.

Gabbet's method is the best for practical purposes; it is very simple, as warming is dispensed with, and decolourising and contrast-staining are done in one operation. The two solutions are also very easy to prepare.

Besides this the method is a very certain one even for sections.

IV. **Czaplewski's Method.**

It is assumed that some of the tubercle bacilli present have become decolourised by the strong mineral acids employed, and so may escape detection. To obviate this, fluorescin is used instead of mineral acids. The method, which applies chiefly to cover-glass preparations, is as follows:—

1. Stain in warm carbol-fuchsine.
2. Pour off the excess of carbol-fuchsine, and place in a strong alcoholic solution of yellow fluorescein containing an excess of methylene blue.

The cover-glass is dipped into this solution six to ten times, allowing the fluid to flow off it again slowly each time.

3. Stain in a strong alcoholic solution of methylene blue.
4. Wash rapidly in water, dry; Canada balsam.

Ehrlich's and Nielsen's methods are especially useful in the case of sections containing quite isolated tubercle bacilli, as the staining may be prolonged if desired.

Leprosy Bacillus.

This closely resembles the tubercle bacillus, and is stained by the same methods.

It differs from it in the fact that it stains much more quickly, but the colour is apt to fade. With sections Gabbet's method gives good results, and according to my experience, fairly permanent preparations. Leprosy bacilli may be distinguished from tubercle bacilli by the fact that they stain with simple aqueous solutions.

Baumgarten's Method.

(1) Stain for six to seven minutes in a dilute alcoholic solution of fuchsine (five drops of a strong alcoholic solution to a large watch glass of water).

(2) Decolourise for a quarter minute in acid-alcohol (nitric acid 1, to alcohol 10).

(3) Wash in water.

(4) Stain in methylene blue; wash in water, etc.; Canada balsam.

The leprosy bacilli are seen as red rods on a blue ground, while the tubercle bacilli are not stained in the short time employed in this method.

As shown by Lustgarten, leprosy bacilli can be differentiated from tubercle bacilli by the fact that leprosy bacilli stained in aniline-water gentian violet or aniline-water fuchsine do not decolourise so readily as tubercle bacilli in a 1 per cent solution of sodium hypochlorite.[1]

Cholera Bacillus.

The cholera bacillus stains very readily in strong aqueous solutions of fuchsine. Ten minutes should be allowed for the staining. It is decolourised by Gram's method. Sections may be stained with fuchsine or methylene blue.

Spirillum of Relapsing Fever (Obermeier).

Günther has devised a method for staining this spirillum in blood. The hæmoglobin is removed from the red corpuscles which do not stain, so that the stained spirilla are better defined.

1. The cover-glass is drawn three times through a flame, or better, is heated for five minutes in an oven at 75° C.
2. Wash for ten seconds in 5 per cent acetic acid.
3. Remove the acetic acid, first by blowing on the cover-glass through a glass tube, and then by holding the cover-glass, with the film, on the under surface, over strong liquor ammoniæ for several seconds.
4. Stain in aqueous solutions of the aniline dyes.
5. Wash in water, dry; Canada balsam.

Nikiforoff's Method.

(1) Harden for twenty-four hours in the following mixture:—

5 per cent aqueous solution of potassium bichromate	equal parts.
Saturated solution of corrosive sublimate in 0·6 NaCl solution . . .	

(2) Continue the hardening in warm 70, 85, 95 per cent alcohol.

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[1] An excellent summary of the various methods for staining B. Lepra and a critique on the differentiation of this from B. Tuberculosis are given by C. Slater, *Quart. Journ. Micros. Science*, 1891.—Ed.

(3) Embed in paraffin.
(4) Stain for twenty-four hours in the following mixture:—

Alcoholic solution of tropæolin . .	5 ccm.
Strong aqueous solution of methylene blue	10 ccm.
Caustic potash solution (1 : 1000) .	2 drops.

(5) Wash in water.

(6) Dip two to three times into a mixture of equal parts of absolute alcohol and ether.

(7) Bergamot oil, xylol, Canada balsam.

Actinomyces.

In examining pus for actinomyces the characteristic whitish granules must be sought for. The pus should be spread out on a glass slide and examined on a dark surface. If granules are found, they should be carefully crushed between a cover-glass and a slide, and on microscopical examination the mycelia and pear-shaped processes or clubs can readily be made out.

Cover-glass preparations can be made and stained in the same way as sections.

A number of methods have been described for staining actinomyces in sections, but they all have the same objection, that they do not invariably give good results, different fungus masses in the same section often staining quite differently. For simply demonstrating the fungus in sections, double-staining with hæmatoxylin and carmine or eosine does very well, the masses being sufficiently clearly differentiated by their red colour from the blue tissue.

Weigert's method for staining bacteria (*vide* p. 85) with previous staining in lithium carmine gives excellent results. The mycelia are better defined by this than by any other method.

Sharp differentiation of the mycelia from the clubs can be obtained by the two following combinations of Weigert's method, and the other parts of the tissue also become well stained.

I. Cover-glass Preparations.

(1) Smear the contents of the follicle on a cover-glass; dry in the air; draw through a flame.
(2) Stain in aniline-water saffranine for twenty-four hours.
(3) Wash in water for a short time.

(4) Stain for five minutes in a saturated aniline-water solution of gentian violet.
(5) Wash quickly in 0·6 per cent salt solution.
(6) Dry between blotting-paper.
(7) Transfer for two minutes to iodine solution (iodine 1, KI 2, water 300).
(8) Dry between blotting-paper.
(9) Decolourise until the preparation ceases to lose colour in aniline oil, which should be repeatedly changed.
(10) Remove the aniline oil with xylol; Canada balsam.

The mycelia stain blue, the clubs brownish red. In this method gentian violet is always used as the second stain.

II. **Sections.**

(1) Stain in aniline-water saffranine for twenty-four to forty-eight hours.
(2) Wash thoroughly in water.
(3) Stain in hæmatoxylin for a half to one minute.
(4) Wash thoroughly in water.
(5) Stain for a half to three hours in a saturated solution of gentian violet in aniline water.
(6) Wash in 0·6 per cent salt solution.
(7) Treat with iodine solution (1 : 2 : 300) for two to five minutes.
(8) Dry on the lifter with blotting-paper.
(9) Decolourise in aniline oil until the section ceases to lose colour.
(10) Stain in an aniline oil solution of eosine for a half-hour.
(11) Remove the aniline oil with xylol; Canada balsam.

The fungus masses are stained dark blue, the clubs brownish red, the nuclei violet, and the protoplasm of the cells rose-red.

Gram's method with twenty-four hours' staining also gives good results, but has a disadvantage as compared with Weigert's method, in that the absolute alcohol dissolves the celloidin, and the follicles drop out to a greater or less extent.

Bostroem recommends the following instead of Weigert's or Gram's method:—

1. Stain in aniline-water gentian violet.
2. Transfer to Weigert's picro-carmine without previous washing.

3. Thoroughly wash in water.
4. Transfer to alcohol, and wash out the gentian violet until the sections become reddish yellow.

The mycelia of the actinomyces follicles remain colourless, the clubs stain red, and the surrounding tissue reddish yellow.

Good sections can be obtained of individual nodules by carefully embedding in celloidin.

Weigert has published a method of staining with orseille.

1. Stain for one hour in a dark-red solution of orseille in the following mixture :—

Absolute alcohol . . .	20·0 ccm.
Acetic acid . . .	5·0 „
Distilled water . . .	40·0 „

2. Wash in alcohol.
3. Stain in a 1 per cent aqueous solution of gentian violet.
4. Wash in alcohol.
5. Xylol, Canada balsam.

The nuclei of the cells are stained a bluish violet, connective tissue orange, the inner part of the ray fungus pale blue, and the outer part ruby red.

Commercial orseille should be left for some days exposed to the air, so that the ammonia it contains shall pass off.

Israel states that the clubs of actinomyces are stained a Burgundy red colour by leaving the sections for some hours in a concentrated solution of orcein in water acidified with acetic acid.

Baranski's Method.

1. Stain the cover-glass preparations or sections for two to three minutes with picro-carmine.
2. Wash for a short time in water.
3. Dry with filter-paper or dehydrate with alcohol.
4. Canada balsam.

The tissue stains red, the centre of the fungus yellow. The clubs do not stain deeply.

Flormann recommends the following process, which closely resembles Kühne's modification of Gram's method (*vide* p. 86).

1. Stain for five minutes in this mixture:—

A strong alcoholic solution of methylene blue	1 part.
Water	2 parts.
1 per cent aqueous solution of ammonium carbonate	2 „

2. Wash for ten minutes in a large volume of water.
3. Place for five minutes in an iodine solution (iodine 1, KI 2, water 300).
4. Wash thoroughly in water.
5. Decolourise by leaving for twenty minutes in an alcoholic solution of fluorescin (one in fifty), which should be changed once.
6. Wash out the fluorescin in 95 per cent alcohol.
7. Place in aniline oil for a few minutes.
8. Oil of lavender.
9. Xylol, Canada balsam.

The mycelia become a dark blue colour, and are very beautifully differentiated. The clubs are partly light blue and partly colourless.

CHAPTER XII

MOULD AND OTHER FUNGI

THE tissue containing the fungus should be teased out in water, or 0·6 per cent salt solution. In this way the various parts of the fungus can generally be observed. If the tissue is not sufficiently transparent, it may be cleared by treating with 1 to 3 per cent caustic potash or soda. Glycerine and alcohol produce considerable shrinkage of the mycelia.

C. Fraenkel's method of teasing in 50 per cent alcohol, to which a few drops of ammonia have been added, is very useful. The material, teased as finely as possible, is afterwards examined in glycerine, and may be preserved permanently by edging round with varnish. Absolute alcohol is generally employed for hardening tissues containing fungi, as it prevents any further growth taking place. Less shrinkage, on the other hand, takes place in Müller's fluid. Vesuvine (Bismarck brown) and methylene blue are best for staining, though the various species behave differently with the different aniline dyes.

Aspergillus stains with fuchsine, methyl-violet, and vesuvine.

Skin fungi or dermatophytes can be examined by **Balzer's** method. The infected hairs and scurf, etc., are first freed from fat by treatment with a mixture of alcohol and ether. They are then stained for a few seconds in an aqueous or alcoholic solution of eosine or in aniline dyes, and after dehydration in alcohol are mounted in Canada balsam. The preparations should be examined in 33 per cent caustic potash if they are not to be kept permanently.

The various forms of fungi occurring commonly in the stomach are best stained with a dilute solution of Bismarck brown, as they very easily overstain with other aniline dyes.

CHAPTER XIII

ANIMAL PARASITES

A ROUGH examination of these parasites requires no special preparation. Many of them, such as acarus scabiei, acarus folliculorum, oxyuris vermicularis, tricocephalus dispar, anchylostomum duodenale, trichina spiralis, distoma hepaticum and lanceolatum, may be examined in water without further trouble. The parasite which is being examined should be flattened out by pressing on the cover-glass with the handle of a needle. The ova of nematodes, cestodes, and trematodes should also be examined in water.

Trichinae in muscle may be examined by teasing. A small fragment of the muscle should be squeezed between two glass slides, and the translucent film which results examined under a low power for the parasite.

Pieces should be taken from the diaphragm and the muscles of the jaw, and preferably portions of muscles near tendons. Sections may be cut with a freezing-microtome with or without embedding in celloidin. The sections should not be cut too thin. Fairly thick sections should be stained with an aqueous solution of methyl-green (1 : 30). In this way the capsule of the parasite is better defined than in thin sections.

Encapsuled and calcified trichinae become transparent on treatment with acids.

Protozoa should be treated with fixing and staining reagents, such as osmic acid, chromic acid, iodine, the aniline dyes, etc.

Protozoa in the contents of the intestine should be examined microscopically at first without treatment, or mixed with salt solution.

Coccidia in hardened tissues stain well with gentian violet and vesuvine. Double - staining in hæmatoxylin and eosine, using a rather stronger solution of eosine than usual, and allowing it to act for several hours, is also an excellent method.

Plasmodia of Malarial Fever.

Fresh blood is examined by **Plehn's method.** A drop of liquid paraffin is placed on a slide, and also on a cover-glass. A drop of blood is caught on the paraffin on the cover-glass, and this is placed on the slide so that the blood lies between a double layer of paraffin. The warm stage is used during the examination. A weak aqueous solution of methylene blue is best for staining blood preparations, a dilute solution of the stain in sterilised ascitic fluid or in blood serum being very suitable. Eosine may be added to this solution for double-staining. For double-staining, the cover-glass preparation, which has been fixed with absolute alcohol, should be treated for five to six minutes with the following solution :—

Strong aqueous solution of methylene blue .	60·0 ccm.
1 per cent solution of eosine in 75 per cent alcohol	20·0 „
Distilled water	20·0 „
20 per cent caustic potash solution . .	12 drops.

Bignami's Method for fixing the Tissues :—

(1) Leave the tissue in the following solution for a half to several hours :—

Corrosive sublimate . .	1·0 grm.
Sodium chloride . .	0·75 „
Acetic acid . . .	0·5 to 1·0 ccm.
Water . . .	100·0 „

(2) Transfer to alcohol containing some tincture of iodine.
(3) Absolute alcohol.

Magenta red (Dr. Grübler, Leipzig) as a saturated aqueous solution, or dissolved in carbolic acid water, is used for staining, and is followed by washing in absolute alcohol.

A mixture of magenta red and aurantia as a saturated alcoholic solution may be used for double-staining.

Taenia.

The heads of tapeworms should be examined under a low power in water, salt solution, or glycerine.

The scolices of echinococcus should be scraped off with a scalpel from the wall of the cyst, and examined in water or dilute glycerine.

The sickle-shaped hooklets may often be seen in teased preparations of dead, and even of calcified echinococci.

By tearing the cyst the scolices of taenia cysticercus are set free, and by squeezing them under a cover-glass the hooklets and suckers may be made out. By compressing a fresh ripe proglottis of a tapeworm between two glass slides, the branched uterus distended with ova is brought into view.

Sections through the wall of an echinococcus cyst made with a razor or a pair of scissors, and examined in water, show very distinctly its characteristic laminated structure.

Methylated spirit is used for preserving and hardening. The cysts become brittle in Müller's fluid. If portions of the tissue become separated in preparing sections, it should be embedded in celloidin. For staining, nuclear dyes are used, either alone or in conjunction with eosine or carmine.

Microscopic preparations may be mounted either in glycerine or Canada balsam. Glycerine is preferable for unstained preparations.

CHAPTER XIV

MICROSCOPIC EXAMINATION OF SPECIAL TISSUES AND ORGANS

BLOOD[1]

Blood may be examined by the following methods :—

(1) In the fresh state, with or without the addition of what is known as a preserving fluid.
(2) As cover-glass preparations, with or without fixing the blood corpuscles by means of a special fixing solution, various staining methods being employed afterwards.
(3) By cutting sections of a fixed and hardened drop of blood and subsequently staining them.

A complete examination entails more than one of these methods.

I. Examination of Fresh Blood.

The best way of doing this is to place a very small drop of blood on the middle of a cover-glass, and to lay this carefully on a cleaned glass slide. Care must be taken to place the smallest possible quantity of blood on the slide, as the finer details of the blood corpuscles can only be made out satisfactorily in a very thin layer.

A good method for obtaining a thin layer is as follows :—A cover-glass is fastened down on a slide with wax smeared round three sides of it. A drop of the blood is allowed to flow under it by capillary action, and the cover-glass is then completely edged round with wax. Blood preparations can be prepared in this way at the bed-side, and examined afterwards at leisure. Plehn's method of mounting the blood between two drops of paraffin (*vide* p. 109) may also be employed. As the blood corpuscles rapidly undergo change in distilled water as well as in acids, and part with their hæmoglobin very readily, a preserving fluid, *i.e.* one that has no action on the corpuscles, should be employed.[1]

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[1] Von Kahlden is indebted for many points in this chapter to H. F. Müller, *Die Methoden der Bluntuntersuchung*, Centralbl. für allg. Path. u. path. Anat. Bd. III.

The following fluids can be used for hardening and preserving blood:—

(1) Salt solution (physiological). This is very useful. The proper degree of strength, varying from 0·6 to 0·75 per cent, must, according to Bizzozero, be ascertained for each species of animal.

(2) Blood serum, lymph, or amniotic fluid from the same species of animal as that under examination. Iodine can be added to the fluid, or a staining solution, such as methyl-green dissolved in 0·6 per cent salt solution, may be run in from the edge of the cover-glass.

(3) A sherry-coloured solution of iodine in potassium iodide is very useful for showing up the outlines of the red blood corpuscles.

(4) A saturated aqueous solution of picric acid has the same property.

(5) 0·5 per cent silver nitrate solution.

(6) **Pacini's Solution**:—

Corrosive sublimate . .	1·0 grm.
Sodium chloride . .	2·0 grms.
Distilled water . . .	200·0 ccm.

(7) **Hayem's Solution**:—

Corrosive sublimate . .	0·5 grm.
Sodium chloride . .	1·0 „
Sodium sulphate . .	5·0 grms.
Distilled water . . .	200·0 ccm.

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[1] In order to examine human leucocytes for their amœboid movements (*e.g.* in blood or pus) Sherrington says that it is not necessary to use a warm stage, because for an hour or so they are very active in an ordinary covered preparation at the temperature of the room, provided that the film is—(α) *sufficiently thin* (the cells, if in contact with both under surface of cover-glass and upper surface of slide, are incited by the mechanical stimulation, and exhibit amœboid movements; the film of blood is thin enough for this if it appears quite transparent, as though "laked," though not really so. If the film is not thin the leucocytes remain spheroids, and motionless); (β) *protected from evaporation*. (Private communication.)—Ed.

The blood is mixed with this solution in the proportion of 1 : 100. After standing for some time the corpuscles sink to the bottom. In twelve to twenty-four hours the fluid is decanted, and the hardened corpuscles thus obtained are washed and filtered. Eosine may be added directly to the hardening solution.[1]

The application of these solutions is limited, as permanent preparations cannot be obtained with them, nor do they prevent with certainty structural changes in the cell nuclei.

These disadvantages may be avoided by using **fixing reagents,** which, however, alter the outlines of the cells.

The following fixing solutions may be mentioned :—

(1) Acetic acid, preferably 1 per cent.
(2) Osmic acid, 1 to 2 per cent.
(3) Flemming's solution (*vide* p. 56), as modified by several authors by substituting a weaker solution of chromic acid.

The staining is done either by adding the stain, such as methyl-green, methyl-violet, saffranine, etc., to the fixing fluid, or by treating sections of the blood subsequently with them (*vide infra*).

II. Cover-glass Preparations.

Aqueous solutions tend to remove hæmoglobin from the corpuscles, a fact which must be borne in mind in staining cover-glass preparations.

Hayem recommends a potassium iodide solution of iodine as a stain which does not dissolve out the hæmoglobin. By treating a film of blood with an iodine solution of a rather deep brown colour, the parts containing hæmoglobin at once become brown or violet. This is the simplest method for detecting the presence of nucleated red blood corpuscles in mammals.

By **Ehrlich's** method, the hæmoglobin may be deprived of its solubility and tendency to swell by heating the cover-glass film, which has already been dried in the air, for one to several hours on a sheet of copper at 120° to 150° C.

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[1] The best results are obtained by adding 10 ccm. of blood to 1 ccm. of normal saline solution, containing 5 per cent neutral potassium oxalate. In this way Sherrington succeeds in keeping leucocytes in the living condition for as long as three weeks. The fluid should be kept in a very cool room or cellar.—Ed.

The same result may be obtained more simply by **Nikiforoff's method.** The cover-glass film, dried in the air, is placed for two hours in a mixture of equal parts of alcohol (absolute) and ether, and is then dried and stained.

Hayem recommends with the same object the action of osmic acid vapour on the dried preparation.

In preparing blood films, the smallest possible drop of blood is placed on one cover-glass and covered with another. When the drop has spread out they are carefully drawn apart. It is essential that the cover-glasses should be held by forceps and not by the fingers, because, as Ehrlich showed, even the moisture of the fingers in the manipulation is sufficient to produce a considerable alteration in the blood corpuscles.

In the examination of blood it is of great importance to be acquainted with the different varieties of white corpuscles which occur in it. According to Ehrlich they are as follows :—

(1) **Small Lymphocytes.**—These are only a little smaller than red blood corpuscles, and have a large, deeply-staining nucleus which occupies the greater part of the cell, so that only a small rim of protoplasm is present around the nucleus.

(2) **Large Lymphocytes.** — These are a further stage in the development of the small lymphocytes. They are two to three times larger than red blood corpuscles, and have also a large nucleus, but this differs from that of the small lymphocytes in being surrounded by a distinct broad mass of protoplasm.

(3) **Mononuclear elements** or **transitional forms** can be distinguished from large lymphocytes by the fact that the nucleus is not uniformly circular, but has a depression in the middle.

(4) **Polynuclear Leucocytes.**—These are smaller than the mononuclear transitional forms, but are larger than red blood corpuscles. They have either a single branching nucleus, or several deeply-staining nuclei. They constitute about 70 per cent of the white corpuscles in normal blood, and possess the power of amœboid movement.

(5) **Eosinophile Cells.**—The nucleus stains less darkly than in the polynuclear leucocytes.

The granules present in the protoplasm (*vide infra*) stain a deep red colour with eosine.

Granules in leucocytes and other cells.—Ehrlich has shown further that the white blood corpuscles contain granules in the protoplasm around the nucleus, which exhibit distinctly different reactions towards certain groups of aniline colours; and that according to their behaviour with dyes, different varieties of granules can be distinguished, which he calls α, β, γ, δ, ϵ granules.

That this depends on true chemical differences follows from the fact that the various granules exhibit other constant features.

(1) In their behaviour towards solvents (water, acids, alcohol, glycerine).
(2) In size, shape, and refractive index.
(3) In their behaviour to raised temperatures.
(4) In the distribution of the granules in the body of the cell.

A. The most important are the **eosinophile cells**—that is to say, cells containing granules which stain deeply with acid aniline dyes, especially with eosine. These are known as α granules. Eosinophile cells may be stained as follows:—

(1) Heat the cover-glass preparation for several hours at 120° C.
(2) Stain for several hours in Ehrlich's acid hæmatoxylin and eosine solution (*vide* p. 45).
(3) Wash in water, dry; Canada balsam.

The nuclei of the lymphocytes and of the polynuclear cells stain darkly; the nuclei of the mononuclear cells bluish gray; the red blood corpuscles copper red, and the eosinophile granules red.

Cover-glass preparations treated by the methods described above may also be stained in the following solution:[1]—

$$\left.\begin{array}{l}\text{Aurantia}\\ \text{Induline}\\ \text{Eosine}\end{array}\right\}\ 2\cdot0\ \text{grms. of each.}$$
$$\text{Glycerine, } 30\cdot0\ \text{ccm.}$$

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[1] For purposes of simple diagnosis cover-glass preparations of the blood, prepared in the ordinary way (*vide* p. 79) by passing the film three times through a flame, are made. These are stained first in eosine (a 50 per cent alcoholic solution of eosine diluted with an equal volume of water), and afterwards in methylene blue.—ED.

Wash in water, dry; Canada balsam.

The nuclei stain blue; the eosinophile cells red or reddish black, and the red blood corpuscles copper red.

For showing the eosinophile cells alone in cases where nuclear-staining is unnecessary, a simple eosine solution is used. A deep red solution of eosine in glycerine is best for this purpose, followed by washing in water.

The presence of these eosinophile cells is, according to Ehrlich, of great importance, who states that they are not increased in number in ordinary acute leucocytosis, but, on the other hand, are very considerably increased in leukæmia.

They only occur in small numbers in normal blood. According to Ehrlich, the fact that the granules become stained by one of the acid dyes is not sufficient to be certain that they are eosinophile granules, and it is necessary to ascertain their colour reactions with the other acid dyes. To make the diagnosis certain the following solutions should be employed:—

(*a*) A deep red solution of eosine in glycerine.
(*b*) A saturated solution of induline in glycerine.
(*c*) A saturated aqueous solution of orange.
(*d*) An eosine-induline-glycerine solution.[1]

B. Basophile Granules.—These stain with the ordinary basic aniline dyes (the bacteria stains, such as methylene blue, gentian violet, fuchsine, Bismarck brown, etc.). The γ granules and δ granules belong to this group. The γ granules are also known as "mastzellen" granules. They do not occur in normal human blood, but are very numerous in leukæmia. The δ granules, which also take up basic aniline stains, are found in the mononuclear transitional forms in human blood.

The basophile γ granules are coarse, the δ granules finer.

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[1] The following is the stain employed by *Demoor* and *Massart* in their recent work on leucocytes:—

Hæmatoxylin .	0·90 grm.
Eosine . .	2·0 ,,
Alum . . .	20·0 ,,
Absolute alcohol	āā 90 ccm.
Glycerine .	
Distilled water	

The results obtained with this solution vary somewhat according to its age. Before mounting in Canada balsam it is as well to examine the depth of staining in each cover-glass, and to increase this, if necessary, by the addition of eosine or hæmatoxylin, as the case may be.—Ed.

Ehrlich recommends the following solution for showing γ granules:—

Glacial acetic acid . .	12·5 ccm.
Absolute alcohol . . .	50·0 „
Water	100·0 „
Dahlia to almost complete saturation.	

Stain for several hours, wash in water, and remove excess of the stain in alcohol.

Cells containing these granules can be stained in sections. The tissue is hardened in alcohol, stained for twelve hours, and decolourised in alcohol.

By **Westphal's method** the nucleus can be stained at the same time by using the following solution:—

Grenacher's carmine (pure carmine, 2·0 grms., alum, 5·0 grms., distilled water, 200·0 ccm. This is boiled, filtered, and 1·0 ccm. carbolic acid added) . .	100 ccm.
Glycerine	100 „
A strong solution of dahlia in absolute alcohol	100 „
Glacial acetic acid . . .	20 „

Wash the cover-glass after staining, allow it to dry in the air, mount in Canada balsam.

The "mastzellen" appear as masses of reddish violet granules, in the middle of each of which is a spot corresponding to the cell nucleus. Nuclei of other cells are stained violet or blue.

According to Westphal, impure commercial methyl-green stains the γ granules bluish violet and the nuclei green.

The δ granules are stained by leaving the cover-glass for ten minutes in a strong aqueous solution of methylene blue, washing in water, drying, and mounting in Canada balsam.

C. Neutrophile Granules or ε Granules.

These stain with neutral aniline dyes[1]—that is to say, with those produced by mixing a basic with an acid dye. They occur closely packed together in the polynuclear leucocytes. They are also found in various mononuclear leucocytes in the blood of myelogenous leukæmia.

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[1] Kanthack and Hardy state that the so-called neutrophile reaction (*i.e.* that produced by what were believed to be neutral dyes) is really an eosinophile (oxyphile) reaction, the "neutral" dyes being, as they show, really acid dyes. —Ed.

Method of Staining.

(1) Prepare a cover-glass film by Ehrlich's method (*vide ante*).

(2) Stain for several minutes in the following mixture:—

Saturated aqueous solution of orange	125 parts.
Saturated aqueous solution of acid-fuchsine containing 20 per cent of alcohol	125 „
To this is added—	
Absolute alcohol	75 „
Saturated aqueous solution of methyl-green	125 „

This solution should not be used until it has been kept for some time. The composition of the solution undergoes changes by filtering, and a precipitate is produced, and it is therefore, in staining, better to take up a drop from the middle of the fluid with a pipette and to place it on the cover-glass.

(3) Wash in water, dry; Canada balsam.

The hæmoglobin stains orange yellow, the nuclei greenish, the eosinophile granules dark gray, the neutrophile granules deep violet.

Sharp definition is obtained by the following method:—

(1) Stain for five minutes in a mixture of

Saturated aqueous solution of acid fuchsine	5 vols.
To which is added, while continually stirring,	
Strong aqueous solution of methylene blue	1 vol.
Distilled water	5 vols.

(2) Wash slightly with water. Draw off the water with blotting-paper. Dry in the air; Canada balsam.

The eosinophile cells stain red, the ε granules violet.

Aronson-Philipp's mixture also gives very good results. Prepare saturated aqueous solutions of

Orange G.
Acid-rubin extra.
Crystals of methyl-green.

These solutions, having been clarified by settling, are mixed as follows:—

Orange G. solution	55 ccm.
Acid-rubin „ (acid fuchsine)	50 „
Distilled water	100 „
Alcohol	50 „
Methyl-green solution	65 „
Distilled water	50 „
Alcohol	12 „

The solution should stand for some weeks. Stain for several hours. Wash in water, dry in the air; Canada balsam. The nuclei stain green, the hæmoglobin orange, the ϵ granules reddish violet, *a* granules yellowish red.

A point of special importance is that these neutrophile or ϵ granules belong to the so-called polynuclear leucocytes, and also to the migratory cells occurring in inflammation. They are found in the greater number of the white corpuscles of normal blood. Pus consists for the most part of these leucocytes with neutrophile granules.[1]

III. Biondi's Method for examining Blood by Means of Sections.

It consists of cutting sections of blood which has been previously hardened. It can be applied to other fluid collections in the tissues.

A drop of blood is placed in 5 ccm. of. a 2 per cent osmic acid solution, and broken up in this by shaking. After a time the cells sink to the bottom. In twenty-four hours (no longer) one to two drops of the osmic acid solution are taken out with a pipette and transferred to 5 ccm. agar-agar, liquid at 33° to 37° C. (Biondi's agar can be obtained from König, 29 Dorotheenstrasse, Berlin.) The osmic acid containing the blood is mixed up with the agar by shaking, and is then poured out into a small paper-box, where it rapidly solidifies. The block of agar is next hardened in 85 per cent spirit, which should be changed several times, and is finally cut between pieces of elder-pith. The sections stain excellently, and the agar, even when deeply stained by aniline dyes, readily decolourises in alcohol. Xylol should not be employed for clearing the sections. Ethereal oils, such as creasote, should be used instead.

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[1] The dyes in general use may be roughly classified as follows:—

(1) *Basic Dyes*—

Fuchsine (all kinds except acid-fuchsine).
Gentian-violet.
Methyl-violet.
Methylene blue.
Bismarck brown (or vesuvine).

(2) *Acid Dyes*—

Acid-fuchsine.
Eosine.
Picric acid.
Hæmatoxylin crystals in aqueous solution.—Ed.

According to Biondi, better sections are obtained by combining the embedding in agar with soaking in paraffin. The block of agar containing the blood is placed for a day in bergamot oil, and is then transferred directly to paraffin at 45° C. for one to two hours. The block is afterwards allowed to solidify under water. The paraffin must be removed before staining. Similar results are obtained by Rindfleisch's and by H. F. Müller's methods.

In **Rindfleisch's method,** the blood, which may be fixed by any process, is washed, mixed with a very small quantity of glycerine, and allowed to evaporate on a glass slide, as much as the glycerine present will permit, and at the same time sheltered from dust. A very thin layer of celloidin is then poured over it, which, after it solidifies, can be removed as a thin film. The film is then stained, etc.

By **H. F. Müller's method** cover-glass films are prepared of blood or other fluids which do not adhere well to the cover-glass. The cover-glass is placed for a moment on a thin celloidin solution, the excess allowed to run off, and then is dried in the air. It is afterwards stained in the ordinary way by Ehrlich's method.

EXAMINATION OF BLOOD PLATELETS.

Blood platelets are flat oval discs, of variable dimensions. They may attain one-third the size of a red blood corpuscle.

It must be borne in mind in investigating them that they undergo considerable changes both in size and shape from slight disturbing causes, even exposure to air.

The process is as follows :—A large drop of 1 per cent osmic acid solution is placed either on one's own skin or on the shaved skin of a warm-blooded animal, and the skin is pricked through this drop. The blood by this means does not come into contact with the air. It is then mixed up with the osmic acid, and the platelets can be satisfactorily examined.

Instead of osmic acid, which was recommended chiefly by Eberth and Schimmelbusch, the following solution may be employed:—

Methyl-violet . . .	0·01 grms.
0·6 per cent salt solution .	50·0 ccm.

Another method for demonstrating blood platelets consists in heating very rapidly a cover-glass film prepared by Ehrlich's method (*vide* p. 113).

The blood corpuscles in dried preparations stain diffusely in strong aqueous solutions of methyl-violet, aniline-green, and fuchsine. When, as generally occurs, a granular centre can be differentiated from a homogeneous peripheral portion, the central part stains somewhat more deeply.

EXAMINATION AND DETECTION OF FIBRIN.

Double-staining with hæmatoxylin and eosine in sections, cut as thinly as possible, is often very successful, as, for example, to show up the network of fibrin in the red hepatisation of pneumonia, and in diphtheritic mucous membranes.

A better method for examining and staining fibrin is that described by **Weigert.** It differs from his method of staining bacteria (*vide* p. 85) only as regards the decolourising fluid used.

Weigert's Method for staining Fibrin.

(1) Harden in alcohol.
(2) Stain for five to fifteen minutes in a strong aniline-water solution of gentian-violet.
(3) Wash in 0·6 per cent salt solution.
(4) Dry with blotting-paper on a lifter or glass slide.
(5) Two to three minutes on a lifter or glass slide in iodine solution (iodine 1, KI 2, water 100).
(6) Dry with blotting-paper.
(7) Decolourise in aniline oil, 2 parts.
xylol 1 part.
(8) Remove the aniline-xylol with xylol.
(9) Canada balsam.

In this way the fibrin becomes stained a beautiful blue colour, whilst everything else except bacteria is decolourised. Remains of blood corpuscles, caseous masses, and coagulation necroses in particular, remain unstained.

A beautiful double stain is obtained by treating the specimen beforehand with lithium carmine (*vide* p. 47).

Fibrin can be stained in fresh preparations of blood by **Löwit's** method. As soon as the blood begins to clot under the cover-glass, 0·6 per cent salt solution is drawn through from the edge until the red blood corpuscles are washed out. The leucocytes and platelets remain behind for the most part. Alcohol is next passed through, and the various processes in Weigert's method are then carried on under the cover-glass.

ABNORMAL CONSTITUENTS OF BLOOD.

Pigment.—This can be satisfactorily examined by mixing up a small drop of blood in a drop of 0·6 per cent salt solution.

Cover-glass preparations can also be prepared either unstained or stained with aniline dyes.

Bacteria.—Anthrax bacilli and the spirilla of recurrent fever are the forms of bacteria which occur most frequently in human blood during life. Anthrax bacilli should be examined either in cover-glass preparations stained by Gram's method, or in drops of fresh blood. The blood of animals infected with anthrax can also be examined very satisfactorily by the hanging-drop method.

In searching for other micro-organisms, especially for cocci, great care must be taken to avoid confusing them with basophile γ and δ granules (*vide* p. 116), which stain like bacteria with basic aniline dyes. δ granules are so minute that they cannot be mistaken for any known form of micrococci. The granules of "mastzellen" may, on the other hand, closely resemble cocci, but are not generally of such uniform size as the latter.

Staining bacteria in dried (cover-glass) preparations of blood is greatly facilitated by treating them for ten seconds with 1 to 5 per cent acetic acid, thoroughly washing, and then staining.

In this way the hæmoglobin is removed from the red blood corpuscles, and the bacteria are stained almost alone. Spirilla of recurrent fever may be examined in fresh blood during the febrile attack. They can be recognised by their active movements.

Dried preparations should be stained in an aqueous or glycerine solution of Bismarck brown or in Löffler's methylene blue (see also p. 102).

COUNTING BLOOD CORPUSCLES.

The blood must be diluted before counting the corpuscles. A pipette is used for this purpose having a sort of bulb in the middle.

This bulb contains exactly 100 times as much as the portion of the tube below it. Blood is first drawn up so as to fill the tube as far as the lower limit of the bulb, and then the diluting fluid is drawn up until the bulb is exactly full. In this way the blood is diluted 100 times.

Various fluids have been recommended for dilution. The examination is easiest with the following :—

Toison's Dilution Fluid.

Methyl-violet . .	0·025 grms.
Neutral glycerine . .	30·0 ccm.
Distilled water . .	80·0 „

To this is added a solution of

Sodium chloride . . .	1·0 grm.
Sodium sulphate . . .	8·0 grms.
Distilled water . . .	80·0 ccm.

The solution is then filtered.

In five to ten minutes the white corpuscles become stained violet, and are well differentiated from red blood corpuscles, which stain a greenish colour.

Thoma-Zeiss's counting apparatus (K. Zeiss, Jena) is now filled up with this fluid. The apparatus consists of a chamber 0·1 mm. deep, the floor of which is divided up into 400 squares,[1] so that the layer of fluid lying on each square contains $\frac{1}{4000}$ ccm.

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[1] The lines engraved on the hæmatocytometer are often insufficiently distinct. The diluted blood approaches more nearly the refractive index of glass than does air, and therefore, though the lines appeared distinct to the maker of the instrument, they tend to give great trouble by disappearing when wanted. The indistinctness may be obviated by carefully pencilling over the lines by rubbing a broad-pointed BB pencil lightly across them, or by rubbing in a little of some aniline dye with a piece of silk.—Ed.

The corpuscles are counted in as many squares as possible, not only those in the squares, but also those on the dividing lines of partition. The calculation is made by multiplying the cubic contents, 4000, by the degree of dilution, and by the number of corpuscles counted, and then dividing the product by the number of squares counted. The result obtained gives the number of corpuscles in 1 cmm. of blood. Expressed by a formula the calculation is as follows:—

$$X = \frac{4000 \times V \times Z}{n},$$

when X = number of corpuscles in 1 cmm. of undiluted blood; V = the extent of dilution, generally 100; Z = number of corpuscles counted lying on the squares; n = number of squares counted.[1]

To estimate the white corpuscles alone, the blood should be diluted by Thoma's method in the proportion of 1 to 10 with water containing 0·3 per cent glacial acetic acid.[2] This dissolves the red corpuscles, and the process of counting is thereby considerably facilitated.

THE HEART AND BLOOD-VESSELS.

The condition of heart muscle can be very well made out by means of teased-out preparations of the fresh tissue, care being taken to tease no more than is necessary.

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[1] Gower's hæmatocytometer, which is very commonly employed in this country for the enumeration of blood corpuscles, will be found described in most physiological text-books. It can be obtained from Hawksley, Oxford Street, W. The Zeiss-Thoma hæmatocytometer is at least twice as accurate as Gower's instrument. A point to remember in using the Zeiss-Thoma instrument is that the cover-glass must lie absolutely flat on the flat glass rim surrounding the cell. If a particle of dust intervenes, the depth of the cell is increased, and the whole estimation falsified. A practical way of ascertaining whether the cover-glass does lie flat is that, if pressed down firmly by the finger, Newton's coloured rings (interference phenomena) appear, and the cover-glass adheres by atmospheric pressure.—Ed.

[2] This rarely gives good results. *Sherrington* recommends the following method for getting rid of the red corpuscles. The graduated slide with the diluted blood is placed on the brass plate of a freezing microtome. The ether spray is used until the fluid just freezes. This is readily seen as a sort of wave which passes over it, and it is immediately allowed to thaw. All the red corpuscles will be found to have disappeared. The red corpuscles may also be got rid of by heating for five minutes at 60° C. In counting leucocytes it is well to add to the fluid used for diluting the blood (preferably 0·3 per cent solution of neutral potassium oxalate in normal saline) enough methylene blue to make the solution a pale blue. This stains the nuclei of the leucocytes, and renders them more visible.—Ed.

To differentiate between cloudy swelling and fatty degeneration, the reagents described in Chapter I. (p. 10) should be used, acetic acid for the former, and 1 per cent osmic acid for the latter (see also p. 63).

Pigmentary degeneration of the cardiac muscle can also be well made out in teased preparations. Müller's fluid is the best for hardening, but alcohol may also be used.

For cutting sections, the tissue should be embedded in celloidin, and the sections afterwards stained with nuclear dyes.

The carmine stains, such as lithium carmine and borax carmine, should be chosen when there is any question of pigment. Picro-carmine defines the transversely striated muscle fibres very distinctly.

Vegetations on valves and elsewhere on the endocardium should be examined in the fresh state for bacteria by means of cover-glass preparations. These should be prepared by finely breaking up the masses on a cover-glass, or by grinding them up in sterilised water with a previously-heated glass rod, and then smearing the fluid on a cover-glass.

Pieces to be cut should be embedded in celloidin, though paraffin may also be employed.

Sections may be stained by simply treating them with gentian violet and washing in alcohol (comp. p. 83); or Gram's method may be used, which stains most of the bacteria present in endocarditic vegetations, especially streptococcus and staphylococcus pyogenes, and Fraenkel-Weichselbaum's pneumonococcus.

To make out the structure of vegetations, sections should be stained with alum carmine, or with hæmatoxylin and carmine or eosine. Weigert's stain for fibrin (*vide* p. 121) is often of use.

The larger blood-vessels are best hardened in Müller's fluid. The methods given below (p. 126) should be employed for showing their elastic tissue.

SPLEEN AND LYMPHATIC GLANDS.

Müller's fluid is the most satisfactory hardening reagent, though corrosive sublimate may be employed. Sections should be stained with nuclear dyes, though Ehrlich's methods for staining blood (*vide* p. 113) can be employed.

For examination of the spleen pulp in the fresh condition, Ehrlich recommends plunging a thick trocar, immediately after death, through the skin into the spleen, and smearing the pulp thus obtained on a cover-glass.

Serous Membranes.

Müller's fluid or alcohol is used for hardening, and the ordinary nuclear dyes for staining.

Serous transudations and exudations, when rich in cells, may be examined as fresh preparations in salt solution.

If the fluid contains few cells, it should be allowed to settle, especially if tubercle bacilli or other bacteria are suspected. This separation may be brought about by centrifugalisation.

The Skin.

Skin should be hardened in Müller's fluid or in alcohol, and may be embedded in celloidin. Alum carmine or any other nuclear dye is used for staining.

Unna's method for showing the elastic fibres in skin is as follows:—

(1) Harden in absolute alcohol, or in Flemming's solution, followed by hardening in absolute alcohol.
(2) Stain with vesuvine (Bismarck brown).
(3) Wash.
(4) Stain for twenty-four hours in the following mixture:—

Fuchsine . .	0·5 grms.
25 per cent nitric acid .	10·0 ccm.
Alcohol . .	āā. 25·0 ccm.
Distilled water . .	

(5) Two to three seconds in 25 per cent nitric acid.
(6) Decolourise in dilute acetic acid.
(7) Dehydrate rapidly in absolute alcohol, cedar-wood oil, Canada balsam.

Herxheimer gives the following method for showing the elastic fibres in skin:—

(1) Harden in Müller's fluid.
(2) Stain for three to five minutes in the following solution:—

Haematoxylin . . .	1·0 grm.
Solution of lithium carbonate saturated in the cold .	1·0 ccm.
Absolute alcohol . .	20·0 „
Water . . .	20·0 „

(3) Decolourise for five to twenty seconds with ferric chloride solution (B.P.).

(4) Wash in water.

(5) Alcohol, oil, Canada balsam.

The elastic fibres stain a bluish black to black, the surrounding tissue light blue, the nuclei of connective tissue cells and round cells light blue to blue. The sections may afterwards be stained with Bismarck brown.

This method cannot be employed for staining in bulk. Absolute alcohol, picric acid, and Flemming's solution may also be used for hardening. Müller's fluid is preferable, however, because decolourisation takes place more readily.[1]

Manchot's method for showing the elastic fibres is as follows:—

(1) Harden in Müller's fluid or in alcohol.

(2) Stain the section for half a minute in a strong aqueous solution of fuchsine.

(3) Remove the excess of the stain in water.

(4) Decolourise for one to twelve hours in a sugar solution of the consistency and fluidity of glycerine, to every 10 ccm. of which three to four drops of sulphuric acid are added.

(5) Mount the sections in non-acidified sugar solution.

The celloidin must be removed from the sections before staining.

Unna-Taenzer's method is very useful.

(1) The tissue should be hardened in alcohol, although Müller's fluid can also be used.

(2) Stain for six to twelve hours in the following solution:—

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[1] The following method of G. E. Goldmann for staining elastic fibres in skin gives very good results (*Beiträge z. klin. Chir.* p. 7, 1893):—

(1) Cut in paraffin.

(2) Fix the section on a slide, remove the paraffin with xylol and place first in absolute alcohol, and then in an alcoholic solution of crystal violet for twenty-four hours.

(3) Decolourise either by Weigert's method for staining fibrin (*vide* p. 121) or in Stroebe's solution used for differentiating axis cylinders (*vide* p. 139). If the former solution be used the aniline-xylol mixture should contain less aniline than that given.—Ed.

Orcein . . .	0·5 grm.
Absolute alcohol . .	40·0 ccm.
Distilled water . .	20·0 „
Hydrochloric acid . .	20 drops.

(3) Decolourise in the following solution :—

Strong hydrochloric acid .	0·1 ccm.
95 per cent spirit . .	20·0 „
Distilled water . .	5·0 „

The demonstration of bacteria in the superficial layers of the skin requires special methods, as the corneous layer possesses the same affinity for basic aniline dyes as bacteria do, so that both are therefore equally affected by overstaining or by decolourisation.

Before staining, the scales of epidermis must be freed from fat. This can be done in several ways :—

(1) The scales are washed in ether and alcohol, stained in an alcoholic solution of eosine, and examined in 33 per cent caustic potash solution (or the staining can be omitted).

(2) The scurf, or the skin itself, is freed from fat in alcohol and ether, stained in an aniline-water solution of fuchsine, washed in acid-alcohol (*vide* p. 47), dehydrated in alcohol, and mounted in Canada balsam. Double-staining with gentian violet may be employed in addition.

By **Bizzozero's method** a cover-glass is gently rubbed on the part of the skin to be examined. It is then passed through a flame three times, and afterwards the fat removed by alcohol and ether.

It is then stained with an aniline dye.

After removal of fat, scurf can be examined by the following method :—

(*a*) Transfer from alcohol to a drop of 50 per cent acetic acid or 10 per cent caustic potash on a slide. As soon as the scales have swollen up, a cover-glass is placed over them, and they can be examined. It is often better to treat the scales on the cover-glass with acetic acid, to evaporate off the acetic acid, and then to treat as a cover-glass preparation and stain with Löffler's methylene blue.

(*b*) The scurf may be examined in glycerine tinged with methylene blue. The bacteria stain blue.

Boeck's method for showing mould fungi in epithelial scales is as follows :—

(1) Remove the fat with alcohol and ether.

(2) Stain for a half to several minutes in Sahli's methylene blue solution, which consists of

5 per cent aqueous solution of borax .	16	parts.
Strong aqueous solution of methylene blue	20	„
Water	24	„

(3) Transfer for a half to one minute to a weak aqueous solution of resorcin (a few particles of resorcin to a large watch-glassful of water).

(4) Leave in alcohol for a few minutes to one hour.

(5) Decolourise in a weak solution of hydrogen peroxide. (This decolourisation is occasionally unnecessary.)

(6) Alcohol, xylol, Canada balsam.

Unna (*Monatshefte f. praktische Dermatol.*, xiii. No. 6, u. 7) gives the following method for detecting bacteria:—

The scales, moistened with a drop of acetic acid, are rubbed up to a pulp between two glass slides. The slides are then separated, and dried rapidly over a flame. A mixture of alcohol and ether is then poured on the obliquely-held slide in order to remove the grease. Stain with borax-methylene blue; wash with water. To decolourise more completely, one of the reagents described by Unna (*vide* pp. 126-128) may be used.

MUCOUS MEMBRANES.

Harden in Müller's fluid. For examination of the epithelium, the tissue should be placed in the hardening fluid in as fresh a condition as possible, and should be embedded in celloidin before cutting. Embedding in celloidin is also necessary for the examination of deposits occurring on the mucous membranes.

The ordinary nuclear stains are used; hæmatoxylin and eosine for double-staining.

THE ALIMENTARY CANAL.

Müller's fluid is the hardening agent chiefly employed. Corrosive sublimate is also suitable. Heidenhain recommends picric acid. For a satisfactory examination of the mucous membrane of the stomach and intestine, it is very important that the pieces of tissue be placed as soon as possible in the hardening solution. In the case of the stomach this can readily be done in the cadaver, by filling it with Müller's fluid through an india-rubber tube immediately after death.

The tissue should be embedded in celloidin before cutting.

The stains used are the ordinary nuclear dyes; hæmatoxylin and eosine for double-staining. The Biondi-Ehrlich stain recommended by Heidenhain (*vide* p. 51) is often very useful.

The contents of the stomach and intestine generally require considerable dilution before they can be examined under the microscope. This is done by taking up the smallest possible quantity of the fluid with a fine platinum loop, and mixing it up on a glass slide with a drop of water or salt solution. The various constituents of fæces can be separated by centrifugalisation.

When blood occurs in the contents of the stomach or intestine, a few red blood corpuscles can generally be found in the tarry mass, which render the diagnosis certain. Failing this, the hæmin tests should be applied (*vide* p. 162).

No special methods are required for the detection of other cell constituents, food detritus, etc. Starch granules can be found by the iodine test (compare p. 66). Cover-glass preparations should be made for the examination of bacteria. Vesuvine is the best stain for the contents of the stomach, as it picks out very distinctly sarcinæ and the different yeast fungi. The ordinary aniline dyes should be employed for the intestinal contents. The contents of the intestine should be largely diluted with sterilised water when examining for bacteria.

It may be pointed out that a number of bacteria staining blue with iodine solution occur in the intestine.

Cover-glass preparations may be made directly from the intestinal contents when searching for cholera bacilli.

Schottelius's method is better. He recommends diluting the intestinal contents with an equal volume of bouillon, and allowing it to stand uncovered. The cholera bacilli grow most abundantly on the surface, so that preparations taken from this contain comma bacilli in larger numbers than would otherwise be the case.

THE LIVER AND PANCREAS.

These organs should be hardened in Müller's fluid. To detect degenerative changes, the liver cells should be examined in fresh scrapings, with the addition of acetic acid or osmic acid (*vide* p. 60).

Flemming's solution (cf. p. 56), or Marchi's solution (*vide* p. 135), should be used as hardening reagents when examining for degenerative changes.

Preparations hardened in Müller's fluid are stained with alum carmine or hæmatoxylin; for double-staining, with hæmatoxylin and eosine.

Tumours should be embedded in celloidin, as the cells are apt to fall out in cutting sections.

Böhm's Method for showing Bile Capillaries.

(1) The pieces of liver, not thicker than 1 ccm., are hardened for seventy-two hours in a mixture of

Three per cent solution of potassium bichromate,	4 vols.
1 per cent solution of osmic acid . .	1 vol.

(2) Transfer for twenty-four to forty-eight hours to a 0·75 per cent solution of silver nitrate.
(3) Wash in distilled water.
(4) Harden subsequently in alcohol. Cut.

The bile capillaries are darkly stained on a yellowish ground.

Böhm's Method for staining the Fibrous Stroma in the Liver:—

(1) Harden pieces of liver, 1 ccm. in size, for forty-eight hours in a half per cent chromic acid solution.
(2) Transfer for seventy-two hours to a 0·75 per cent silver nitrate solution.
(3) Leave for several hours in distilled water.
(4) Harden in alcohol. Cut.

The fibrous tissue stains black.

The fibrous stroma in preparations hardened in alcohol may also be stained by Oppel's method.

Oppel's Method.

(1) Transfer the pieces of liver from alcohol to a 10 per cent aqueous solution of yellow chromate of potassium for twenty-four hours.
(2) Transfer to 0·75 per cent silver nitrate solution. The volume of this solution should be twenty to thirty times that of the pieces of tissue.
(3) Change the silver nitrate solution in one hour's time, and again after two to three hours.
(4) Wash for twenty-four hours in distilled water.
(5) Transfer to alcohol. Cut.

The tissues should not be embedded in paraffin, as this renders them brittle.

URINARY ORGANS.

Harden in the same way as the liver and pancreas. Corrosive sublimate also in many cases gives good results.

The method of boiling (*vide* p. 16) should be employed for fixing albuminoid fluids in the glomeruli and tubules. Moderate sized pieces of kidney are plunged in boiling water for one to two minutes, and afterwards hardened in alcohol. The same result is produced by hardening in absolute alcohol, and embedding in celloidin. The same methods are employed for investigating degenerative changes, as in the liver and pancreas. Embedding in celloidin is indispensable in minute examination, as otherwise portions of the secreting tubules always drop out, especially in kidneys which have undergone morbid changes. For staining, the nuclear dyes are used.

Urine.—After standing, the sediment is taken up with a pipette, is examined on a slide, or cover-glass preparations may be made. The latter method is used more especially for the detection of bacteria.

Tubercle bacilli, in cases of tubercular disease of the urinary tract, are generally only found in small numbers in the urine. The urine should be allowed to stand for twenty-four hours, and a considerable number of cover-glass preparations, six to ten, or even more, made from the sediment, and stained by some of the methods already described. The centrifugal machine may also be used with advantage. Gabbet's (*vide* p. 100) method is one of the best on account of its simplicity, and the certain results it gives. In doubtful and important cases it is often useful to stain the cover-glasses in an incubator for twenty-four hours with aniline-water fuchsine.[1]

It is often better, when examining urine for cells, etc., to dilute the sediment with water or salt solution. Acetic acid gives the cells a sharper definition. They may also be rendered more distinct by allowing a drop of Löffler's methylene blue to flow in from the edge of the cover-glass.

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[1] **Van Ketel's method** for tubercle bacilli in the urine:—

(1) Add carbolic acid to the urine in the proportion of 1 to 20 parts urine.

(2) Shake vigorously for at least five minutes.

(3) Allow to settle in a conical glass.

(4) Draw off some of the sediment with a fine pipette.

(5) Make cover-glass preparations by heating in the ordinary way.

(6) Dip the cover-glass several times in chloroform (or a mixture of equal parts of alcohol and ether), which renders it transparent.

(7) Stain with ordinary carbol-fuchsine.

This is a very reliable method, and is also applicable for sputum.—Ed.

Permanent specimens may be made from cover-glass preparations stained with Löffler's methylene blue or Bismarck brown.

The examination of casts is rendered much easier by suspending the sediment in a dilute iodine solution of a pale yellow colour.

Blood in urine can generally be detected by direct microscopical examination, as at least a few red corpuscles will probably be seen. In doubtful cases the tests for hæmin (*vide* p. 162) should also be applied.

The sediment should be examined fresh for crystals.

Acid urate of sodium, which forms a brick-dust coloured deposit when present in large quantities, is amorphous.

Uric acid is generally found in the form of "whetstone" crystals, rhombic plates, or long pointed needles.

Urate of ammonium is formed by decomposition of the urine, and occurs as "hedgehog" crystals.

Ammonium-magnesium phosphate (triple phosphates) are oblong crystals ("coffin-lids").

Calcium oxalate occurs as square envelope-shaped crystals.

Calcium carbonate forms spherical and flat bodies.

Bilirubin occurs in both the amorphous form and as yellow rhombic plates.

Cystin forms regular hexagonal plates.

Tyrosin forms sheaf-like bundles of needles, and **leucin** spherical bodies.

THE RESPIRATORY ORGANS AND SPUTUM.

Hardening in Müller's fluid is best. The tissues should be hardened in alcohol when fibrin or bacteria are to be sought for. A mixture of gum and glycerine (*vide* p. 15) is useful when rapid hardening is necessary. To fix inflammatory exudations or œdematous tissue the boiling method (p. 16) should be applied, followed by hardening in strong alcohol. Embedding in celloidin should be employed in all cases in which abnormal contents of the pulmonary alveoli have to be dealt with.

The ordinary nuclear stains are employed, and, in addition, the special bacterial stains and Weigert's stain for fibrin can be used.

Sputum can either be examined undiluted, or after the addition of salt solution, though this is generally not required. In the examination of sputum it is important to bear in mind that it contains cells from the mouth and pharynx, and that *débris* of food may also be mixed up with it.

Besides salivary corpuscles, squamous epithelium cells from the mouth, round cells, and mucous cells, there may also be found in saliva large cells resembling epithelial cells, but of a rounder shape. They have a large round nucleus, and are considered, although not in all cases, to be desquamated (pulmonary) alveolar epithelium cells. They often contain pigment. They occur in ordinary bronchitis (catarrhal cells).

Pigment.—Cells containing pigment granules may originate from hæmorrhages, especially from chronic pulmonary congestion due to mitral disease. Red blood corpuscles can often be found as well as the pigment.

The pigment resulting from hæmorrhage has to be distinguished from that derived from inspired air.

Except in the case of siderosis, adventitious pigment gives no iron reaction with potassium ferrocyanide and hydrochloric acid (p. 71), and is generally black, while blood pigment is brownish red. The difference of colour alone, however, is not diagnostic.

Fibrinous casts from the bronchi can be recognised by their characteristic shape.

So-called **Asthma crystals** (Charcot) are long, very pointed octahedra.

Curschmann's spirals are ribbon-shaped spiral bodies with a clear thread in the centre. Crystals of the fatty acids occur in fetid bronchitis, gangrene, and abscess of the lung, and in cavities.

Many varieties of **micro-organisms** are found in all sputa.

Tubercle bacilli and elastic fibres occur most abundantly in small, caseous, plug-like masses in the sputum. These are the portions, therefore, which should be most carefully examined when searching for either of these abnormal constituents (*vide* examination of sputum, p. 98).

The cover-glass method is employed for the examination of bacteria. Elastic fibres are detected by either treating the fresh sputum with 1 per cent caustic potash, or by boiling it with 10 per cent caustic potash. It is then allowed to settle, and the sediment examined in twelve to twenty-four hours.

THE CENTRAL NERVOUS SYSTEM.

Müller's fluid is employed almost exclusively for preserving and hardening pieces of tissue from the central nervous system. The spinal cord takes three to four months to harden properly, and the brain four months to a year. The time required for hardening can be considerably shortened by placing it in an incubator. The tissue can be kept in Müller's fluid for several years, provided that when the hardening is completed, the solution is diluted to one-half by the addition of water. The pieces may be kept in spirit for some length of time after this. They should be placed in the preserving solution as soon as possible after removal.

It is sometimes advisable not to place the tissue at first in Müller's fluid of the ordinary strength, but in one containing only 1 per cent of potassium bichromate.

Hardening in Flemming's solution is very useful in the examination of degenerative changes in nervous tissues.

The following method is particularly suitable for detecting slight degenerative changes :—

Marchi's Method.

(1) The pieces, which should be as small as possible, are kept in Müller's fluid for eight days (or longer if preferred).[1]

(2) Place them for six to eight days in the following mixture :—

Müller's fluid . .	2 parts.
1 per cent osmic acid .	1 part.

(3) Wash carefully in water.

(4) Harden in alcohol of increasing degrees of strength.

(5) Embed in celloidin.

In removing the parts from the body great care should be taken to avoid dragging on them.

Parts which have undergone degeneration appear black; everything else is light gray. The tissue may be afterwards stained with carmine; preferably with lithium carmine (5 per cent); the staining takes several hours. Weigert's method of staining may also be applied, if the preparations are put back again into Müller's fluid for a sufficiently long time.

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[1] Four weeks is generally best.—Ed.

Teased preparations of portions of the central nervous system freshly taken from the body can only be prepared with difficulty and are unsatisfactory. The teasing is easier after three to eight days in Müller's fluid.

There are many different staining methods for the central nervous system. They either stain the nuclei alone, or the axis-cylinders, or the medullary sheaths.

I. The simple nuclear stains show all that is required in many cases, and are specially suitable for small inflammatory foci.

II. **Nissl's Method for staining Ganglion Cells.**

Nissl describes two methods, both of which give good results:—

Method a.

(1) Harden in alcohol of successively increasing strengths. The pieces of tissue should be about a half cm. thick.
(2) Stain the sections in a strong aqueous solution of fuchsine, which should be heated until vapour arises from the surface.
(3) Wash in absolute alcohol for one to two minutes.
(4) Clear in oil of cloves until no more colour is given out.
(5) Canada balsam.

Method b.

(1) Harden as in (a).
(2) Stain in 0·5 per cent methylene blue solution, which should be heated until it bubbles and begins to boil.
(3) When the staining solution has cooled down, wash the sections in the following mixture:—

Aniline oil . .	20·0 ccm.
90 per cent alcohol .	200·0 „

This should be continued as long as the colour comes out, and until the white and gray matter are clearly differentiated from each other.

(4) Remove the section on a slide, dry with blotting-paper, and cover it with a drop of origanum oil, which should be allowed to flow off again in a short time.

(5) Dry with blotting-paper, and afterwards remove with benzine any origanum oil which remains.

(6) Cover the section with a solution of resin in benzine, evaporated down to the consistency of thin Canada balsam.

(7) Pass the slide through a flame which ignites the benzine resin.

(8) Cover with a cover-glass, and warm slightly until the benzine-resin completely fills the space between slide and cover-glass.

III. Axis-cylinder Staining by Van Giesson's Method.

The easiest and most certain method for demonstrating these structures is **Van Giesson's** (p. 54). The axis-cylinders stain a brownish-red colour, the medullary substance yellow, the nuclei bluish red, the neuroglia red, the nerve-cells with their processes red, and all sclerosed tissue a brilliant red. The method gives better differentiation and more certain results than were formerly obtained by the use of carmine, and on this account is an excellent general stain.

Specimens hardened in alcohol can also be stained by this method, though Müller's fluid is preferable, as the differentiation is more marked and the yellow staining of the medullary sheath better defined.

IV. Axis-cylinder Staining with Neutral Carmine.

Neutral carmine (*vide* p. 52) often gives good results. It is best to leave the sections in a dilute solution for a long time, up to twenty-four hours, and then to wash them thoroughly. Neutral carmine stains the axis-cylinders, nerve-cells, and interstitial tissue. Degenerated areas appear a deep red colour.

The time required for staining varies considerably according to the age of the preparation and the method by which it has been hardened. The staining can be hastened and deepened by placing the sections for ten minutes in a palladium chloride solution (0·01 in 50), and transferring directly to the carmine solution. Warming also aids the staining. In all cases, however, when time allows, it is best to stain slowly in a dilute solution.

The nerve-cells are best stained by neutral carmine, by leaving the sections for twenty-four hours in a light rose-coloured solution.

V. **Borax Carmine** followed by treatment with acid-alcohol (*vide* p. 47) gives good results. The sections should be left in the stain for a quarter to several hours, though no precise time can be stated. The parts stained are the axis-cylinders, interstitial tissue, nuclei of nerve-cells, and other nucleated structures.

Lithium carmine gives similar results, and is employed in the same way.

VI. Czokor's Cochineal Stain for Axis-cylinders.

Cochineal . . .	1·0 grm.
Alum . . .	1·0 „
Water . . .	100·0 ccm.

This is boiled down to half its volume.

Stain for twenty-four hours. Wash in water.

The nuclei are stained violet, the axis-cylinders a redder colour.

VII. Schmaus's Carmine Stain for Axis-cylinders.

(1) Harden in Müller's fluid without washing afterwards.
(2) Harden in absolute alcohol in the dark.
(3) Stain in the following solution:—

Sodium carminate . .	1·0 grm.
Uranium nitrate . .	0·5 „
Water . .	100·0 ccm.

This is rubbed up and dissolved in the water, boiled for half an hour, and filtered when cold.

(4) Wash in water, etc.

Schmaus also recommends an axis-cylinder stain consisting of a blue-black solution (0·25 grms. in 100·0 ccm. 50 per cent alcohol), to which some picric acid is added. Stain for one hour. Wash in water.

VIII. Stroebe's Aniline-blue Stain for Axis-cylinders.

To stain the axis-cylinders and medullary substance simultaneously, **Ciaglinski** recommended, first staining with saffranine, followed by washing in water, staining with aniline-blue, then washing in water. Stroebe has improved this method by washing in alkaline-alcohol instead of water, after staining with aniline-blue.

Stroebe's Method is as follows :—

(1) Harden in Müller's fluid.
(2) Stain for a half to one hour in a saturated aqueous solution of aniline-blue.
(3) Wash the blue-black sections in water.
(4) Place them in a dish containing alcohol, to which twenty to thirty drops of caustic-potash alcohol have been added. (Caustic potash 1 grm. to alcohol 100 ccm.; allow to stand twenty-four hours, then filter.) The sections are left in this until they become a light brownish-red colour and transparent. One or more minutes are generally sufficient.
(5) Transfer for five minutes to distilled water. The sections become light blue again.
(6) Contrast-stain for a quarter to half an hour in a saturated solution of saffranine diluted to one-half with water.
(7) Wash and dehydrate in absolute alcohol. Xylol; Canada balsam.

IX. Sahli's Method.

(1) Harden carefully in Müller's fluid.
(2) Stain the section for ten minutes to several hours in the following mixture :—

50 per cent borax solution . .	16·0 ccm.
Saturated aqueous solution of methylene blue	24·0 ,,
Distilled water . . .	40·0 ,,

(3) Wash in water or alcohol until the gray matter has a distinct light blue colour against the dark blue of the white matter.

(4) Clear with cedar-wood oil; Canada balsam. The medullary sheaths stain deep blue, the nerve-cells a pale greenish colour, and the nuclei of the neuroglia cells blue. This method is specially adapted for showing at the same time any bacteria which may be present. The specimens cannot be kept for an unlimited time.

X. **Freud's gold Chloride Method** of staining the axis-cylinders alone.

(1) Harden in Müller's fluid.
(2) Stain for two to five hours in a mixture of equal parts of 1 per cent gold chloride solution and 45 per cent alcohol.
(3) Wash in water.
(4) Leave for two to three minutes in this solution:—

Caustic soda	. .	1·0 grm.
Distilled water	. .	6·0 ccm.

(5) Wash in water.
(6) Five to ten minutes in a 10 per cent potassium iodide solution.
(7) Wash; alcohol; Canada balsam.

Freud's gold stain does not always prove successful.

The axis-cylinders stain dark blue to dark red. The medullary layer often stains to a certain extent at the same time. Glass needles should be used for manipulating the sections.

There are many modifications of the gold chloride method. **Cohnheim,** who was the first to employ it for showing the nerves in the corneous layer of the skin, places the sections in 0·5 per cent gold chloride solution, and then for some days in water acidified with acetic acid.

The modifications of the method lately recommended consist of the following:—

(*a*) The use of much more dilute solutions in which the sections are left for a correspondingly longer time.
(*b*) The use of the double chloride of gold and potassium or sodium instead of gold chloride.
(*c*) The changing of the gold chloride solution.
(*d*) By reducing with hydrochloric acid, formic acid, or tartaric acid instead of acetic acid.

The gold chloride method is much more successful with fresh than with hardened tissues.

XI. **Upson's gold Chloride Method** for axis-cylinders.

(1) Harden for four to six months in a solution of potassium bichromate in distilled water, which should be repeatedly changed. The strength of the solution should at first be 1 per cent, afterwards 2 per cent. The hardening process should be carried on in the dark.

(2) Wash the hardened pieces of tissue as rapidly as possible in distilled water.

Leave them for two to three days in 50 per cent alcohol, prepared by diluting absolute alcohol. This must be repeatedly changed.

(3) Leave them in 95 per cent alcohol (prepared from absolute) until they exhibit a distinct green colour; two to four weeks are generally required for this.

(4) Embed in celloidin; cut.

(5) Stain the sections for two hours in the following solution:—

Gold chloride . . .	1·0
Hydrochloric acid . . .	2·0
Distilled water . . .	100·0

(6) Wash the sections in distilled water.

(7) Transfer to a 10 per cent caustic potash solution for half a minute.

(8) Wash in water.

(9) Reduce in the following solution, which must be renewed for each section:—

Sulphuric acid . . .	5 ccm.
3 per cent tincture of iodine	10 to 15 drops.

Mix and add one drop of liquor ferri chloridi.

(10) Wash in water as soon as the section has turned a rose-red colour.

(11) Alcohol; oil of cloves; Canada balsam.

The sections should be kept in the dark. All manipulations should be done with platinum or glass needles. Contact with metals must be avoided.

XII. Method of staining Axis-cylinders with Nigrosine.

(1) Stain the sections in a strong aqueous solution of nigrosine for five to ten minutes.
(2) Decolourise, first in dilute and then in absolute alcohol.
(3) Origanum oil, Canada balsam.

This method is useful, owing to its simplicity. It gives a very good general idea of the position of any tracts of degeneration that may be present.

The following is the best method for staining the medullary sheath of nerve-fibres :—

XIII. Weigert's Hæmatoxylin Method of staining the Medullary Sheaths.[1]

(1) Harden in Müller's fluid.
(2) Harden afterwards in alcohol without previously washing in water.
(3) Embed in celloidin.
(4) Leave the block of celloidin containing the tissue for twenty-four to forty-eight hours in a half-saturated solution of acetate of copper.
(5) Twenty-four hours in 70 per cent alcohol.
(6) Cut.
(7) Stain for fifteen or twenty minutes to twenty-four hours in the following solution :—

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[1] Application of Weigert's method to sections prepared by the freezing methods. *N.B.* It is difficult in some cases to cut sections of large area, *e.g.* pons or medulla, by the paraffin or celloidin method.

The tissue is hardened in Müller's fluid, and afterwards in alcohol, as in (1) and (2).

(3) Transfer direct from the alcohol to a saturated aqueous solution of acetate of copper ; leave it in this overnight in an incubator at 40° C.

(4) Soak for three to twelve hours, according to the size of the piece, in the following solution of mucilage. The mucilage is of B.P. strength, but in place of water a saturated aqueous solution of copper acetate is used.

(5) Freeze and cut.

(6) Remove the sections from the razor to saturated copper acetate solution, to get rid of the gum, and leave them in this for ten minutes.

(7) Transfer to 70 per cent methylated spirit. If allowed to remain in this for twenty-four hours at least, on transference to the Weigert hæmatoxylin solution the sections turn an ink black in a few seconds. The rest of the process is as usual.—Ed.

Hæmatoxylin	1·0 grm.
Saturated solution of lithium carbonate	1·0 ccm.
Absolute alcohol	10·0 „
Distilled water	60·0 „

(8) Wash in a large volume of water.
(9) Partially ~~dehydrate~~ decolourize in this solution :—

Sodium biborate	4·0 grms.
Potassium ferricyanide	5·0 „
Distilled water	200·0 ccm.

The time required for decolourising cannot be given exactly. It should be continued until the gray matter acquires a distinctly yellow colour.

(10) Wash in water.
(11) Dehydrate in absolute alcohol.
(12) Clear in xylol.
(13) Mount in Canada balsam.

The pieces of tissue hardened in Müller's fluid should still be a brown colour, and should not have become green in the alcohol. If this occurs they should be transferred for a few minutes or longer to a $\frac{1}{2}$ per cent solution of chromic acid, washed very slightly, and placed in the stain at once. The pieces of tissue or the sections may also be replaced for some time in Müller's fluid.

Weigert's method gives very good results; the white matter stains a deep blue-black colour, degenerated parts stain lightly in proportion to the number of nerve-fibres which have undergone change. Any medullary sheaths remaining among the destroyed nerve-fibres also often take up the stain.[1]

The time required for staining with hæmatoxylin varies considerably. Spinal cord sections often stain in fifteen to thirty minutes. Sections of the cerebral cortex require up to twenty-four hours, and it is advisable to place the staining fluid for at least part of the time in an incubator. If the sections are taken out of the stain too soon, some of the fibres remain unstained, and deceptive appearances may thus result.

It is often advisable to decolourise more slowly by diluting the ferricyanide solution to a half, or even a quarter the strength.

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[1] Weigert's method XIII. often shows up very clearly fatty degeneration in cardiac muscle.—Ed.

If origanum oil is used for clearing, the sections should not be left in it longer than is absolutely necessary, or decolourisation will result.

Should it be found desirable to stain a portion of the tissue by some other method, a piece should be cut from the celloidin block before placing it in the copper solution. Instead of placing the entire piece of tissue in the copper solution (4), the separate sections may be treated with the solution after cutting.

XIV. **Weigert's Method** of staining white matter without differentiation.

This modification described by Weigert is as follows:—

(1) Harden as in method XIII.
(2) Transfer to a mixture of

Saturated solution of copper acetate . . } equal parts,
10 per cent solution of potassium sodium tartrate }

and leave it in an incubator for twenty-four hours.

(3) Transfer to a mixture of

Saturated solution of copper acetate } equal parts,
Water . . . . }

for twenty-four hours in an incubator.

(4) Stain in the following solution:—

Hæmatoxylin . . .	1·0 grm.
Absolute alcohol . . .	10·0 ccm.
Saturated solution of lithium carbonate .	7 „
Distilled water . . .	90·0 „

(This should be prepared by adding the last two to the alcohol and hæmatoxylin solution.)

(5) Wash in water.
(6) Clear in the following mixture:—

Aniline oil	2 parts.
Xylol	1 part.

The sections may be transferred to this mixture straight from 90 per cent alcohol.

(7) Mount in xylol-Canada balsam.

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If the celloidin is stained too deeply, the sections should be passed through 1 to 5 per cent acetic acid after staining. If the sections themselves are overstained, the potassium ferricyanide decolourising fluid described above should be employed.

XV. **Pal's Method.**

This is a useful modification of Weigert's method.

(1) Harden in Müller's fluid, etc.
(2) Stain in Weigert's hæmatoxylin solution for twenty-four to forty-eight hours, for the last hour in an incubator.
(3) Wash in water, to which 1 to 2 per cent of lithium carbonate has been added. The sections should be stained a deep blue.
(4) Transfer the sections for twenty to thirty seconds to a 0·25 per cent solution of permanganate of potash until the gray matter appears yellow.
(5) Transfer for a few seconds to the following solution :—

Pure oxalic acid . . .	1·0
Potassium sulphite . . .	1·0
Distilled water . . .	200·0

(6) Wash thoroughly in water.
(7) Alcohol, xylol, Canada balsam.

The advantage of this method is that the separate processes can be carried out much more rapidly. It gives very sharp definition. The tissue between the nerve-fibres being completely decolourised, it may be counter-stained, for which purpose picro-carmine or borax carmine is best adapted.

XVI. **Vassale's** modification of Weigert's method.

(1) Harden in Müller's fluid, afterwards in alcohol.
(2) Stain for three to five minutes in this solution :—

Hæmatoxylin . . .	1·0 grm.
Water	100·0 ccm.

(Dissolve with the aid of heat.)

(3) Transfer for three to five minutes to a saturated filtered solution of copper acetate.
(4) Wash quickly in water.
(5) Transfer the sections to the following solution :—

Borax	2·0 grms.
Potassium ferricyanide	2·5 „
Water	300·0 ccm.

(The sections should be kept moving to and fro in this solution.)
(6) Wash thoroughly in water.
(7) Alcohol, carbolic-acid xylol (1 : 3), Canada balsam.

In order to obtain good results the hæmatoxylin solution must be fresh.

XVII. **Kulschitzky's Method** for staining the medullary sheaths.

(1) Harden in Müller's fluid, or, better, in Erlicki's fluid (*vide* p. 14). (In the latter the tissue should remain for one to two months, and be afterwards hardened in alcohol.)
(2) Stain for eighteen to twenty-four hours in the following solution, which should be slightly acidified with acetic acid before using.

Hæmatoxylin	1·0 grm.
Saturated solution of borax	20·0 ccm.
Distilled water	80·0 „

(3) Wash in water.

Kulschitzky's staining fluid is yellow at first, but in two to three weeks it turns a deep red, and is then ready for use. Before using it, a few drops of acetic acid should be added to a large watch-glassful of the fluid.

The medullated nerve-fibres become stained a deep blue colour; everything else remains unstained or takes up a faint yellowish tint. This stain is much improved by leaving the sections for twenty-four hours in a saturated solution of sodium or lithium carbonate.

A more recent method published by Kulschitzky is as follows :—

(1) Stain for one to three (rarely twenty-four) hours in

Hæmatoxylin (dissolved in absolute alcohol)	1·0
2 per cent acetic acid . .	100·0

(2) Decolourise in the following solution:—

Saturated solution of lithium carbonate	100 ccm.
1 per cent potassium ferricyanide solution	10 ,,

(This generally takes two to three hours.)

(3) Wash thoroughly in water.

A similar result is obtained by boiling for one to four hours 2 grms. (or more) of carmine in 100 ccm. of a 10 per cent acetic acid solution. The sections are left in this for two to four hours, and are then placed direct in the decolourising fluid given above (2).[1]

XVIII. **Exner's Method** for showing medullated nerve-fibres.

The tissue should be taken from the body as soon as possible, though the method succeeds even when twelve hours have elapsed after death. Pieces of the brain or spinal cord, not more than one-half cm. thick, are placed at once in a 1 per cent osmic acid solution, the volume of which should be at least ten times that of the tissue.

The osmic acid solution is renewed in two days' time. In five or six days the pieces are washed in water, and are either cut directly after fixing on a cork, or after previous embedding in

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[1] **Kulschitzky** gives the following method for staining neuroglia (*Anat. Anzeiger Centralbltt.*, 1893 [357]).

(1) Harden in the dark for two to three months in Erlicki's fluid (*vide* p. 14).
(2) Harden subsequently in the dark in absolute alcohol.
(3) Embed in paraffin; cut.
(4) Stain in the following solution:—

Patent acid rubin . .	0·25 parts.
2 per cent acetic acid solution . .	100 ,,
Saturated aqueous solution of picric acid . .	100 ,,

Take three to five ccm. of this mixture and add 100 ccm. of 96 per cent alcohol. Leave the sections in the stain for half an hour or longer.

(5) Wash in two portions of 96 per cent alcohol. The sections should be left in the second for some hours.

(6) Transfer to absolute alcohol; clear; mount.—Ed.

celloidin. Each section is at once mounted in glycerine, and one drop of ammonia solution (liquor ammoniæ 1 to water 50) is added to the glycerine on the slide.

The medullated nerve-fibres appear a gray or black colour by Exner's method; the specimens are, however, not permanent.

XIX. **Adamkiewicz's Method.**

Adamkiewicz has pointed out that saffranine stains the medullary sheath of nerve-fibres red, and, on the other hand, the nuclei of the nerve-cells and neuroglia cells and those in the vessel walls violet.

The medullary sheaths of degenerated nerve-fibres, however, do not stain. This holds even in the earliest stages of degeneration.

The method is as follows:—

(1) Harden in the various solutions containing chromium salts.

(2) Transfer the sections for a short time to water slightly acidified by the addition of a few drops of nitric acid.

(3) Stain in a deep red aqueous solution of saffranine No. 0. The sections do not readily overstain in this.

(4) Wash in ordinary alcohol.

(5) Transfer to absolute alcohol slightly acidified by the addition of nitric acid.

(6) Clear in oil of cloves until the colouring matter ceases to come out.

(7) Mount in Canada balsam.

By this method the medullary sheaths stain yellowish red or red, and the nuclei of the connective tissue cells bluish violet.

The results obtained by this method are by no means so constant as stated by Adamkiewicz. **Nikiforoff's** modification of the method is an improvement.

(1) Harden in solutions of chromium salts.

(2) Complete the hardening process in alcohol, without previous washing in water.

(3) Place the sections, after cutting, directly in alcohol.

(4) Stain for twenty-four hours in a strong aqueous solution of saffranine, or in aniline-water saffranine, or carbolic acid water (5 per cent) saffranine.
(5) Wash carefully in alcohol, moving the section to and fro until the gray matter is differentiated from the white by its lighter staining.
(6) Transfer to gold chloride solution (1 : 500) until the gray matter has a slight violet tinge.
(7) Wash carefully in water.
(8) Transfer to absolute alcohol until the gray matter is clearly marked out by its violet colour from the white matter which is stained red.
(9) Oil of cloves for a short time.
(10) Xylol.
(11) Canada balsam.

XX. **Golgi's Nitrate of Silver Method** for showing nerve-cells and their processes.

The pieces of tissue, which should be small, are hardened in Müller's fluid, and then transferred directly from this to a 0·75 per cent silver nitrate solution, which should be changed in an hour's time. The pieces may remain as long as desired in the fresh solution.

In five to six days the tissue, after being dried slightly, is placed first in dilute, and then in absolute alcohol, and afterwards is cut.

Alcohol, oil, Canada balsam.

The ganglion cells and their processes stain black, and also the connective-tissue cells. The method is very unreliable, as the nerve-cells rarely all take up the stain. Golgi's corrosive sublimate method is an improvement.

XXI. **Golgi's Corrosive Sublimate Method** for nerve-cells and their processes.

The piece of tissue, not more than 0·5 cm. thick, is hardened in Müller's fluid, and then placed in 0·25 per cent corrosive sublimate solution, which should be changed as long as it becomes tinged yellow. Small pieces may be cut in eight to ten days' time, though it is better to leave them longer in the solution.

The sections should be very thoroughly washed.

Alcohol, oil, Canada balsam.

By this method also the nerve-cells with their processes stain black, but this method, like the foregoing, is an uncertain one.

An improvement suggested by Pal consists in treating the sections afterwards for some minutes with a solution of sodium sulphide (Na_2S).

Specimens prepared by the corrosive sublimate method are very delicate, and cannot be preserved under a cover-glass. This is also the case with the silver preparations.

Sections can be permanently mounted and double-stained by the following method :—

XXII. **Obregia's Method.**

(1) Corrosive sublimate or silver nitrate preparations prepared by Golgi's method are treated with alcohol (of strength not less than 95 per cent). They are then placed for half an hour in the following solution :—

1 per cent ferric chloride solution	8 to 10 drops.
Absolute alcohol . . .	10 ccm.

The solution should be freshly prepared and exposed to diffused light for half an hour.

(Metal needles, etc., should not be used.)

The sections should be left in this solution for fifteen to thirty minutes in the dark.

(2) Wash quickly in 25 per cent alcohol, then in distilled water.

(3) Place in 10 per cent sodium sulphide solution for five to ten minutes (not longer).

(4) Wash repeatedly in distilled water.

(5) Stain with carmine or hæmatoxylin by Weigert's, Pal's, etc., methods. Mount in Canada balsam, cover with a cover-glass.

The following is a slight variation of Kölliker's modification of Golgi's method :—

XXIII. **Ramon y Cajal's Method.**

(1) The pieces of fresh tissue are left for twenty-four, thirty-six, or forty-eight hours in the dark in the following solution :—

Potassium bichromate . .	3 parts.
1 per cent osmic acid solution .	25 „
Distilled water . . .	100 „

The fluid should be changed after some hours, but may be used again for the preliminary treatment of other pieces of the tissue.

(2) Treat with a 0·25 per cent silver nitrate solution for a quarter to half an hour.
(3) Transfer to a 0·75 per cent silver nitrate solution for thirty-six to forty-eight hours.
(4) Wash in 40 per cent alcohol.
(5) Dehydrate quickly in absolute alcohol.
(6) Cut by hand between elder-pith, etc. (*vide* p. 27).

Embedding in paraffin and celloidin cannot be employed. Cover-glasses should not be placed on the specimens.

These methods, though useful, cannot always be relied upon and the staining is not uniform.

Nerve-cells and processes, and all non-medullated nerve-fibres stain deep black; the neuroglia cells and their processes stain reddish black.

XXIV. **Flechsig's Modification of Golgi's Corrosive Sublimate Method.**

Flechsig devised this method for demonstrating the connection between the processes of the nerve-cells with the reticulum in the gray matter, and further, the connection of the reticulum with medullated nerve-fibres.

The first brain examined by this method was hardened in Müller's fluid, and was then left for a whole year in 1 per cent corrosive sublimate.

(1) Harden in a 2 per cent solution of potassium chromate.
(2) Soak in corrosive sublimate solution.
(3) Cut. The sections should be placed in 96 per cent alcohol.
(4) Stain for three to eight days at 35° C. in the following solution :—

Pure extract of Japanese red wood	1 grm.
Saturated solution of sodium sulphate	5 ccm.
„ „ tartaric acid	5 „
Absolute alcohol	10 „
Distilled water	900 „

(5) Transfer each section separately to 3 ccm. of $\frac{1}{4}$ to $\frac{1}{5}$ per cent solution of potassium permanganate until the fluid loses its colour.

(6) Decolourise in the following solution:—

Oxalic acid	1·0 grm.
Potassium sulphite	1·0 „
Distilled water	200·0 ccm.

If decolourisation is not complete, repeat again (5) and (6) until the yellow tint has vanished from the section.

(7) Transfer the section to a mixture of

1 per cent gold chloride solution	5 drops.
Absolute alcohol	20 ccm.

until the mercurial precipitate which was a whitish colour by reflected light becomes a deep black, and the red bundles of nerve-fibres acquire a bluish tint.

(8) Wash for a short time in

5 per cent potassium cyanide solution	1 drop.
Distilled water	20 ccm.

The sections should be floated on the fluid.

(9) Dehydrate in absolute alcohol.

(10) Clear in pure oil of lavender.

(11) Canada balsam.

The bundles of nerve-fibres are stained a carmine red; the nerve-cells and processes a deep black colour.

XXV. **Wolter's Method** for staining axis-cylinders.

(1) Harden in the dark for twenty-four hours in the following mixture:—

Sulphate of copper Potassium bichromate	any quantity desired.

50 per cent alcohol to each 100 ccm., to which six drops of acetic acid should be added.

(2) Transfer to absolute alcohol for twenty-four hours. Embed in celloidin. Cut.

(3) Transfer the sections for twenty-four hours to a mordant composed of

Vanadium chloride . .	2 parts.
8 per cent ammonium acetate solution . . .	8 „

(4) Wash for ten minutes in water.

(5) Stain for twenty-four hours in an oven in hæmatoxylin . . . . 2·0 grms.
Absolute alcohol (sufficient to dissolve).
3 per cent acetic acid . . . 100·0 ccm.

(6) Wash in 80 per cent alcohol acidified with hydrochloric acid until the sections acquire a light bluish-red colour.

(7) Wash out the acid with dilute alcohol; dehydrate in absolute alcohol; origanum oil; Canada balsam.

The nerve-cells and neuroglia stain as well as the nerve-fibre.

XXVI. **Malbory's Method for Staining Axis-cylinders.**

Stain the sections for ten minutes to one hour in the following solution :—

Phospho-molybdic acid .	1 part.
Hæmatoxylin . .	1 „
Chloral-hydrate .	6 to 8 parts.
Water . . .	100 „

This solution should be exposed to sunlight for a week, and filtered before using.

(1) Wash in 50 per cent alcohol, which should be changed two to three times.

(2) Dehydrate, etc.

The stained sections should not be left too long in alcohol. The amount of hæmatoxylin in the staining fluid may often be increased with advantage.

PERIPHERAL NERVES AND GANGLIA.

These are in general treated by the same methods as the central nervous system. Good preparations can often be obtained by teasing.

Mays has described the following method for showing non-medullated nerves and nerve-endings in muscle.

Mays's Method.

(1) The pieces of tissue are thoroughly soaked in a 5 per cent solution of arsenic acid (As_2O_5).

(2) Transfer for ten minutes to the following solution:—

1 per cent solution of the double chloride of gold and potassium .	4·0 ccm.
2 per cent osmic acid .	1·0 „
0·5 per cent arsenic acid .	20·0 „

(3) Wash in water.

(4) Transfer to a 1 per cent solution of arsenic acid, and expose to sunlight for three hours on a water-bath at a temperature of 45° C. in this solution.

(5) Clear in the following mixture:—

25 per cent hydrochloric acid	1·0 ccm.
Glycerine . . .	40·0 „
Water . . .	20·0 „

The method of injecting the tissues with methylene blue has lately been extensively used for demonstrating peripheral nerves and ganglia. The principle was first employed by Ehrlich, but has since been considerably modified.

Methylene Blue Injection Method.

(1) A 4 per cent methylene blue solution (preferably Dr. Grübler's "Rectificertes methylen blau zur vitalen injection"), having been previously filtered, is injected at once, or as soon as possible after death, into the blood-vessels of the organs the nerve-structures of which are to be examined.

The organ which has been thus treated is left exposed to the air until it becomes blue. This point is determined by teasing out fresh pieces of the tissue.

(2) As soon as the colouration has become general, the colour is fixed by placing the tissue in a cold saturated aqueous solution of ammonium picrate, which should be filtered before using. If sections are to be cut, the tissue is placed for twenty minutes to twelve hours, according to the size of the piece, in a cold saturated alcoholic solution of ammonium picrate. The following fixing solution is sometimes better:—

Saturated aqueous solution of ammonium picrate	100 ccm.
1 per cent osmic acid . . .	2 „

(3) Sections may be cut between pieces of lardaceous liver, elder-pith, etc. (*vide* p. 27), or with the freezing microtome.

Sections or teased preparations should be mounted in a mixture of equal parts of glycerine and water, to which a trace of ammonium picrate is added.

If other constituents of the tissue, such as cement substance, nerve cells, blood corpuscles, etc., become stained at the same time, the nervous tissues are so well defined that they are quite unmistakable.

Degeneration changes in peripheral nerves can be detected by hardening in Flemming's or in Marchi's solutions, by which means degenerated parts of the medullary sheaths are stained black.

Obersteiner has recently recommended Platner's method with ferric chloride and dinitro-resorcin for showing degeneration in axis-cylinders.

Platner's Method.

(1) Harden the nerve-trunks for one to several days in the following solution:—

Liquor ferri sesquichloridi	1·0 ccm.
Distilled water . .	4·0 „

(2) Wash in water until the washings cease to give a reaction with potassium sulphocyanide.

(3) Transfer the pieces of nerve to a solution prepared by adding an excess of dinitro-resorcin to 75 per cent alcohol for several weeks, according to their size.

(4) Dehydrate; embed; cut.

By the combination of the iron solution and dinitro-resorcin the axis-cylinders acquire an emerald-green colour, and changes in them are very clearly defined.

The method can be applied to preparations which have been hardened in Flemming's solution or in Müller's fluid.[1]

---

[1] *Degeneration of peripheral nerves.* I am indebted to Dr. Sherrington for the following details, which are of considerable practical importance :—When trying to determine whether any of the fibres remain normal, or whether all are degenerated, it should be borne in mind that this point cannot be ascertained by transverse sections, because, although all the fibres may be undergoing degeneration, many of them will still appear normal in cross-section, even when the degeneration is advanced (*e.g.* three to four weeks after section of the nerve-trunk). The degenerating fibres break up into short lengths, and in many places the transverse section of these pieces is indistinguishable from the transverse section of a normal fibre. It is necessary, therefore, to cut longitudinal sections, preferably a short series in paraffin, or to tease out a piece of the fresh nerve.

For treating peripheral nerves ·5 per cent osmic acid is better than 1 per cent, as it penetrates better, and renders them less brittle.—Ed.

THE EYE.

For preparing sections of the entire eye Müller's fluid and Flemming's solution are the only hardening reagents employed. Eyes can be kept for a very long time in Müller's fluid. Embedding in celloidin is necessary for a detailed examination of the eyeball. By this means the relative position of the layers is preserved. Staining in bulk with Bismarck brown (p. 49) or Beale's carmine (*ibid.*) is sometimes advisable, so as to minimise the risk of disturbing the relation of the different parts. Embedding in paraffin may also be employed.

Hæmatoxylin and alum carmine are used for nuclear-staining. The retina and optic nerve should be treated by the various methods already described for the central nervous system (*vide* p. 135). Double-staining is often useful.

Cohnheim's gold chloride method (p. 140) should be employed for the cornea. The cornea, taken from a freshly-killed animal, is placed for five minutes in fresh filtered lemon juice. It is then transferred for twenty minutes to a 1 per cent gold chloride solution, and, lastly, is exposed to the light for three to four days in water slightly acidified with acetic acid. It is embedded in celloidin and cut in alcohol.

THE EAR.

Portions of the middle and inner ear should be hardened in Müller's fluid. For the external ear alcohol may be used as well. Decalcification of the adjacent bony tissues, when necessary, is postponed until the hardening process is completed.

OSSEOUS SYSTEM.

Bones and joints are hardened in Müller's fluid. Preparations hardened in alcohol do not give such good results. Decalcification should be done after complete hardening. Preparations hardened in Müller's fluid are first thoroughly washed, left in alcohol for some days, and then decalcified. (For decalcifying methods *vide* p. 17.)

Stain with hæmatoxylin; double-staining with hæmatoxylin and neutral carmine gives very good results, as the carmine stains decalcified bones and ossifying cartilage red, but not tissue which has undergone calcareous infiltration.

In this process the sections stained in hæmatoxylin are washed for twelve to twenty-four hours in water before placing them in carmine.

The ground substance of decalcified cartilage from which the lime salts have been dissolved stain deep bluish violet with hæmatoxylin.

Unaltered cartilage varies in its behaviour to neutral carmine and hæmatoxylin; resting cartilage generally stains better with carmine, while, on the other hand, growing and proliferating cartilage stains deeper with hæmatoxylin.

The cells of the marrow of bones are very well defined by double-staining with hæmatoxylin and eosine.

Pommer has published a method for studying the structural arrangement of bone, and for detecting areas in bones which contain no lime salts. The method depends on a peculiar action of Müller's fluid. Bones left in Müller's fluid for a long time become capable of being cut, owing to the feebly decalcifying action of its acid salts. In addition to this, however, it differentiates between calcified and non-calcareous portions. This is not the case with bones decalcified by acids. The method is as follows:—

Pommer's Method.

The bones should be kept in Müller's fluid until they can be cut easily with a sharp razor.

In the sections from bone thus prepared the decalcified bony matter is clearly differentiated by its homogeneous appearance from the parts which have not undergone calcification, and which are conspicuous by their distinctly fibrillar structure.

By treating with carmine the detection of small non-calcareous areas is much facilitated, and the relations of the different parts in sections become much plainer.

Pommer recommends one of the following solutions for staining bones which have been decalcified in acid mixtures, such as Von Ebner's fluid:—

(1) Dahlia . .	0·04 per cent solution.
(2) Saffranine . .	0·01 to 0·16 per cent solution.
(3) Methyl-green .	0·30 per cent solution.

The decalcified parts stain rather deeply in twelve to eighteen hours in any of these solutions, and rather more with saffranine and dahlia than with methyl-green.

The parts which do not contain calcareous material, on the contrary, remain quite colourless, and contrast most distinctly with the parts which previously contained lime salts.

Scheffer has also recommended a method for staining bony tissues with saffranine.

Scheffer's Method.

(1) Decalcify with nitric acid or with an acidified sodium chloride solution.
(2) Stain for a half to one hour in an aqueous solution of saffranine (1 in 2000).
(3) Wash in water.
(4) Transfer to a 0·1 per cent corrosive sublimate solution.
(5) Examine in glycerine.

Contact with alcohol should be avoided as far as possible. To prepare permanent specimens, therefore, the sections, after treatment with corrosive sublimate (4), should be rapidly passed through alcohol, and the greater part of the water removed by pressure with blotting-paper; the rest can be got rid of by leaving them for a long time in bergamot oil. They can then be mounted in Canada balsam.

MUSCLES, TENDONS, SYNOVIAL SHEATHS, AND BURSAE.

These should be hardened in Müller's fluid. Sections of muscle cannot be prepared satisfactorily without embedding in celloidin. Picro-carmine (p. 48) is a very suitable stain for muscle; it stains the protoplasm yellow. Hæmatoxylin and alum carmine may also be used, or double-staining with hæmatoxylin and carmine or eosine.

Picric acid (p. 54) also is useful for double-staining.

MALE AND FEMALE SEXUAL ORGANS.

Harden in Müller's fluid. Embed in celloidin, especially the ovary and placenta.

To examine fragments of the uterine mucous membrane for carcinoma, the pieces should be hardened in alcohol and embedded in celloidin. It is advisable to fix several pieces on one cork and cut them all at the same time. This is readily done by surrounding the cork with paper so as to form a box (*vide* p. 23), and in this way several sections from several different fragments can be examined at once in one preparation.

The examination is thus rendered much easier and more rapid, as very often the first portion, which has been cut by itself, may contain no part suitable for examination. There is also a much greater chance of cutting sections containing the muscular tissue immediately underlying the mucous membrane. Single-staining with hæmatoxylin is used in these cases.

Shreds of decidua and remains of placenta can be examined in the same way.

CHAPTER XV

MICROSCOPICAL EXAMINATION FOR MEDICO-LEGAL PURPOSES

Detection of Blood.

In stains on wood, metal, or clothing, the examination should be directed to—

(1) The recognition of red blood corpuscles.
(2) Obtaining evidence of the presence of blood pigment.

I. To find blood corpuscles in recent stains some of the scrapings are soaked in 0·6 per cent salt solution (distilled water should not be used), and examined directly under the microscope.

The blood corpuscles of man and of mammals are circular and without nuclei, those of other animals being oval and nucleated, so that so far the examination determines whether, on the one hand, the stain is due to the blood of man or mammals, or, on the other hand, to that of some other class.

Human red blood corpuscles are larger than those of other mammals. The diameter is 0·0077 mm. Following them in order of size come the red corpuscles of the dog, rabbit, pig, ox, horse, cat, and, lastly, the sheep.

Human can be distinguished with fair accuracy from other mammalian blood corpuscles by measurement with a micrometer. As many corpuscles as possible should be measured to ensure accuracy. An absolute diagnosis is prevented by the very rapid changes in shape and form of the corpuscles. In older blood stains, red corpuscles which have retained their characteristic shape may, however, often be found. Generally, simple salt solution is not sufficient for the examination, and other fluids have to be employed. The following are the most useful:—

(1) 30 per cent caustic potash. In using this care must be taken not to dilute with water.

(2) **Roussin's** fluid.

Glycerine	3 parts.
Strong sulphuric acid	1 part.

(3) **Pacini's** fluid (modified).

Corrosive sublimate	1·0 grm.
Sodium chloride	2·0 grms.
Glycerine	100·0 ccm.
Water	300·0 ,,

These fluids are used in the following manner :—

A portion of the stain is scraped off, or, in the case of clothes or material, is removed, placed on a slide, and the fluid added to it. The blood corpuscles as they become visible are examined at once under the microscope, as they gradually undergo changes in the macerating fluid.

In old blood stains in which the corpuscles have undergone much more shrinking, differences in size of the corpuscles are much less obvious than in recent cases.

Great care must be taken to avoid mistaking the spores of some of the moulds for corpuscles. Spores resist very strongly the action of acids and alkalies.

II. The detection of blood pigment by the preparation of hæmin crystals is a more reliable test for blood.

Hæmin, the hydrochlorate of hæmatin, is a derivative of hæmoglobin. Hæmin crystals are known also as Teichmann's blood crystals, after their first observer.

They can be prepared from dried blood as follows :—

If the blood stains are on a hard substance such as wood, they are carefully scraped off, and the red mass collected on a glass slide or in a watch-glass, and dissolved in a small quantity of distilled water.

After removal of extraneous matter, etc., the reddish coloured fluid is evaporated down and a drop of 0·6 per cent salt solution, the size of a pin's head, added. This is spread out on the surface of the slide and again dried. When this is done, the dried mass is scraped up with a scalpel, and glacial acetic acid added with a glass rod (the test is very uncertain with dilute acid). A cover-glass is then placed on it, and it is heated over a spirit-lamp until the acetic acid begins to bubble. Gentle warming is continued until the acetic acid is evaporated. When this has taken place the brown hæmin crystals separate out. Larger hæmin crystals are obtained the more slowly the evaporation has been carried on. Evaporation on this account should be performed under a cover-glass.

Hæmin crystals are quite insoluble in water, ether, and alcohol. They are soluble with difficulty in ammonia, dilute sulphuric acid, and in nitric acid, but readily dissolve in caustic potash. They form small rhombic plates.

The presence of fatty substances often hinders crystallisation; in such cases it is advisable to treat the material beforehand with ether.

The presence of rust from iron may also hinder the separation of hæmin crystals.

EXAMINATION OF HAIR.

The first point to decide in all medico-legal examinations is whether the hair in question is human or from an animal.

With this in view, attention must be paid to the following microscopical features:—

(1) The outermost layer of the hair, the cuticle, is composed of fine scales of epidermis lying upon one another like slates on a roof, with their pointed ends directed towards the free end of the hair. If these cells cannot be clearly made out without further treatment, dilute nitric acid should be added. In most animals the cells of the cuticle are much larger than in man, so that they are much more clearly defined; and in many animals the cuticle cells stand out more, so that they give a much more serrated appearance to the hair than is the case in man.

(2) The middle layer or cortical substance is composed of elongated corneous cells. These are rendered more distinct by the addition of dilute nitric acid.

In human hair the cortical substance greatly exceeds in breadth the innermost layer of medullary substance. In animals the proportion is quite the reverse.

(3) The cell structure of the medulla in human hair is very indistinct, while in animals it can readily be made out. In

human hair the medulla is often wanting, and when present its continuity is frequently interrupted. This is rarely the case with animals, and when it occurs is always limited to quite isolated hairs. It is obvious that the hair should be examined throughout its whole length.

It is advisable in each individual case, in order to decide whether the hair is human or from some animal, to compare it not only with human hair, but also with hair from such animals as are ordinarily met with.

Characteristics of Hairs from Different Parts of the Body.

Hairs from the beard are usually the thickest, 0·14 to 0·15 mm. in diameter; then follow in order female pubic hairs, hairs from the eyelashes, male pubic hairs, hairs from the male scalp, and, lastly, hairs from the female scalp, about 0·06 mm. in diameter. The frequency of individual variations should always be borne in mind. Differences due to age are also very considerable, the hairs of new-born children being distinctly thinner than those of older children, and especially of adults.

Hairs of new-born children have a pointed extremity, as have also hairs which have undergone no disturbance during their natural growth, such as cutting, pressure from clothing, softening from perspiration, etc. Cut hairs at first exhibit a sharp section, but later have a more rounded termination.

Hairs which have been pulled out generally have an open, club-shaped root, with the remains of the hair sac; hairs which have dropped out have a closed, smooth, atrophied root.

To decide whether the hair in question comes from a particular individual, methods of comparison should be employed, attention being paid to the size of the entire hair as well as of the various layers, and as to colour, etc. For this purpose sections can be cut with a razor; embedding in paraffin (*vide* p. 24) is preferable.

Detection of Seminal Stains.

In examining stains for spermatozoa it should first be ascertained whether fine scales can be separated from the more or less firmly adherent patches on the material. Such scales, which must be very gently handled on account of their brittleness, should be mixed upon a glass slide with a drop of distilled water, and then teased up with a pair of needles. The specimen is then examined under a high power with a narrow diaphragm.

If the stain has soaked deeply into the material so that scales cannot be separated from it, a small portion should be cut out and macerated for a quarter to half an hour in a watch-glass containing distilled water. By adding a small quantity of hydrochloric acid (1 drop to 40 ccm. water), the spermatozoa are prevented from swelling up. The piece of stuff is then squeezed by pressing on it with the handle of a needle, and the milky fluid thus obtained may then be examined directly.

The examination can also be carried on by teasing a small piece of the material on a glass slide in water. It is very essential that the stuff should have been well soaked beforehand.

If spermatozoa are found, cover-glass preparations may be obtained in the ordinary way, and stained with neutral carmine or eosine. Hæmatoxylin and eosine are used for double-staining.

Unger recommends a special staining solution :—

Methyl-green . .	0·15 to 0·3 grms.
Water . . .	100·0 ccm.
Hydrochloric acid .	3 drops.

This solution may be used for soaking the piece of material under examination, and it is then examined directly under the microscope. It may also be employed for staining the cover-glass preparations. The hinder part of the head stains dark green, the anterior portion light green, while the median part and the tail stain a lighter colour than the hinder part of the head.

EXAMINATION OF DECIDUAL REMAINS.

This may be necessary in cases where there is suspicion of induced abortion. The decidua of pregnancy can be distinguished from all other constituents of tissues by the characteristic large polygonal or rounded cells.

Fresh teased preparations may be made, but it is better to cut small sections after hardening in alcohol. These can be cut either by hand or in a cylinder microtome (*vide* p. 32). After surrounding with paraffin, they can also be cut between fresh elder-pith or hardened liver (*vide* p. 27).

They stain best with hæmatoxylin.

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